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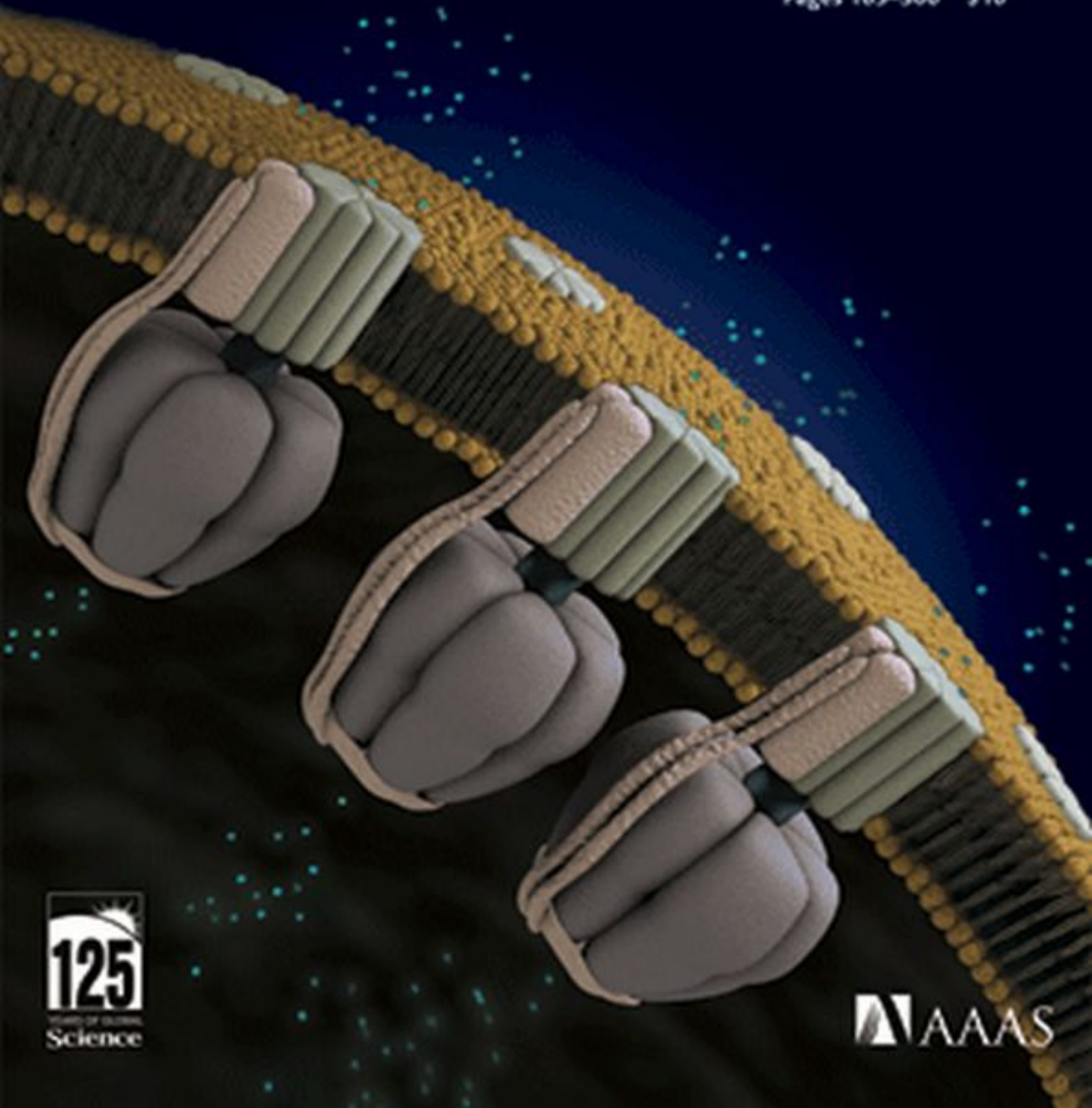

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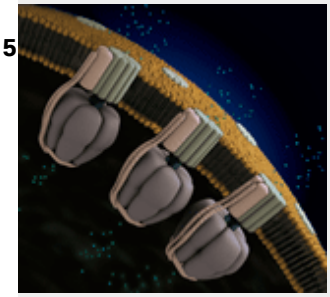


NEW THIS WEEK:

Science

Testing Hypothesis Strength
New Drug Hope for TB
Spintronic Theory Confirmed
Hormone Determines Heart Size
Epilepsy Drugs Lengthen Worm Life-Span

Research
- This Week in Science
- Reviews
- Brevia
- Research Articles
- Reports

News
- News Summaries
- News of the Week
- News Focus

Commentary
- Editorial
- Letters
- Policy Forum
- Book Reviews
- Perspectives

- Editors' Choice
- NetWatch
- ScienceScope
- Random Samples
- New Products
- Science Online Contents

RESEARCH

This Week in Science

Caged Gas * New Tool for the TB Armory * Variation on a Theme * Spin Switching Nanomagnets * Tuning Superatom Chemistry * A Tamed Radical * Resolved Bump * An Albatross's Life * Retinoic Acid and Heart Development * Gut Antigen Sampling and Host Defense * Anticonvulsant Medications and Aging in Worms * Another Route to Stat Regulation * Testing the Strength of Hypothesis * Directions Home * A Clock by Another Mechanism * Form and Function? 177

Editors' Choice: Highlights of the recent literature

APPLIED PHYSICS: Chip-Scale Magnetic Measurements * NEUROSCIENCE: Making Memories * CLIMATE SCIENCE: Twinned Thinning * CHEMISTRY: Maintaining Chains * ECOLOGY/EVOLUTION: Eats Roots or Shoots * BIOTECHNOLOGY: Library Science * STKE: Specificity Through Degradation 182

Review

Testing Hypotheses: Prediction and Prejudice
Peter Lipton 219-221.

Brevia

The First Glacial Maximum in North America
Greg Balco, Charles W. Rovey, II, and John O. H. Stone 222.

Research Article

A Diarylquinoline Drug Active on the ATP Synthase of *Mycobacterium tuberculosis*
Koen Andries, Peter Verhasselt, Jerome Guillemont, Hinrich W. H. Göhlmann, Jean-Marc Neefs, Hans Winkler, Jef Van Gestel, Philip Timmerman, Min Zhu, Ennis Lee, Peter Williams, Didier de Chaffoy, Emma Huitric, Sven Hoffner, Emmanuelle Cambau, Chantal Truffot-Pernot, Nacer Lounis, and Vincent Jarlier 223-227.

Reports

Time-Domain Measurements of Nanomagnet Dynamics Driven by Spin-Transfer Torques
I. N. Krivorotov, N. C. Emley, J. C. Sankey, S. I. Kiselev, D. C. Ralph, and R. A. Buhrman 228-231.

Al Cluster Superatoms as Halogens in Polyhalides and as Alkaline Earths in Iodide Salts
D. E. Bergeron, P. J. Roach, A. W. Castleman, Jr., N. O. Jones, and S. N. Khanna 231-235.

A Stable Aminyl Radical Metal Complex
Torsten Büttner, Jens Geier, Gilles Frison, Jeffrey Harmer, Carlos Calle, Arthur Schweiger, Hartmut Schönberg, and Hansjörg Grützmacher 235-238.

Encapsulation of Molecular Hydrogen in Fullerene C_{60} by Organic Synthesis
Koichi Komatsu, Michihisa Murata, and Yasujiro Murata 238-240.

Corrected Late Triassic Latitudes for Continents Adjacent to the North Atlantic
Dennis V. Kent and Lisa Tauxe 240-244.

An Astronomical 2175 Å Feature in Interplanetary Dust Particles
John Bradley, Zu Rong Dai, Rolf Erni, Nigel Browning, Giles Graham, Peter Weber, Julie Smith, Ian Hutcheon, Hope Ishii, Sasa Bajt, Christine Floss, Frank Stadermann, and Scott Sandford 244-247.

Retinoic Acid Signaling Restricts the Cardiac Progenitor Pool
Brian R. Keegan, Jessica L. Feldman, Gerrit Begemann, Philip W. Ingham, and Deborah Yelon 247-249.

Global Circumnavigations: Tracking Year-Round Ranges of Nonbreeding Albatrosses
John P. Croxall, Janet R. D. Silk, Richard A. Phillips, Vsevolod Afanasyev, and Dirk R. Briggs 249-250.

No Transcription-Translation Feedback in Circadian Rhythm of KaiC Phosphorylation
Jun Tomita, Masato Nakajima, Takao Kondo, and Hideo Iwasaki 251-254.

CX_3CR1-Mediated Dendritic Cell Access to the Intestinal Lumen and Bacterial Clearance
Jan Hendrik Niess, Stephan Brand, Xiubin Gu, Limor Landsman, Steffen Jung, Beth A. McCormick, Jatin M. Vyas, Marianne Boes, Hidde L. Ploegh, James G. Fox, Dan R. Littman, and Hans-Christian Reinecker 254-258.

Anticonvulsant Medications Extend Worm Life-Span
Kimberley Evason, Cheng Huang, Idella Yamben, Douglas F. Covey, and Kerry Kornfeld 258-262.

Self-Propagating, Molecular-Level Polymorphism in Alzheimer's ß-Amyloid Fibrils
Aneta T. Petkova, Richard D. Leapman, Zhihong Guo, Wai-Ming Yau, Mark P. Mattson, and Robert Tycko 262-265.

Semaphorin 3E and Plexin-D1 Control Vascular Pattern Independently of Neuropilins
Chenghua Gu, Yutaka Yoshida, Jean Livet, Dorothy V. Reimert, Fanny Mann, Janna Merte, Christopher E. Henderson, Thomas M. Jessell, Alex L. Kolodkin, and David D. Ginty 265-268.

Stat3 Dimerization Regulated by Reversible Acetylation of a Single Lysine Residue
Zheng-long Yuan, Ying-jie Guan, Devasis Chatterjee, and Y. Eugene Chin 269-273.

COMMENTARY

Editorial

The Science of Social Diseases
Christopher Dye 181.

Letters

Retraction *Owen N. Witte, Janusz H. Kabarowski, Yan Xu, Lu Q. Le, and Kui Zhu* ; Scientific Priorities in North Korea *Courtland Robinson, Myung-Ken Lee, Gilbert Burnham;, and Norman P. Neureiter* ; North Korea and Renewable Energy *David F. Von Hippel and Peter Hayes* ; Inflammation and Life-Span *Calogero Caruso, Giuseppina Candore, Giuseppina Colonna-Romano, Domenico Lio, Claudio Franceschi;, Anthony G. Payne;, Caleb E. Finch, and Eileen M. Crimmins* 206.

Policy Forum

ECOLOGY:
The Convention on Biological Diversity's 2010 Target
Andrew Balmford, Leon Bennun, Ben ten Brink, David Cooper, Isabelle M. Cue, Peter Crane, Andrew Dobson, Nigel Dudley, Ian Dutton, Rhys E. Green, Richard D. Gregory, Jeremy Harrison, Elizabeth T. Kennedy, Claire Kremen, Nigel Leader-Williams, Thomas E. Lovejoy, Georgina Mace, Robert May, Phillipe Mayaux, Paul Morling, Joanna Phillips, Kent Redford, Taylor H. Ricketts, Jon Paul Rodríguez, M. Sanjayan, Peter J. Schei, Albert S. van Jaarsveld, and Bruno A. Walther 212-213.

Books et al.

HISTORY OF SCIENCE: Lost in Translation?
Stuart McCook 210-211.

MARINE ECOLOGY: Voice of the Turtle
Fredric J. Janzen 211.

Perspectives

MICROBIOLOGY: Enhanced: TB--A New Target, a New Drug
Stewart T. Cole and Pedro M. Alzari 214-215.

APPLIED PHYSICS: A Ringing Confirmation of Spintronics Theory
Mark Covington 215-216.

CHEMISTRY: Odd Electron on Nitrogen: A Metal-Stabilized Aminyl Radical
Wolfgang Kaim 216-217.

CELL SIGNALING: Stat Acetylation--A Key Facet of Cytokine Signaling?
John J. O'Shea, Yuka Kanno, Xiaomin Chen, and David E. Levy 217-218.

NEWS

News of the Week

ATMOSPHERIC SCIENCES: NOAA Loses Funding to Gather Long-Term Climate Data
Jeffrey Mervis 188.

RAINFALL MONITORING: Report Bucks NASA's Plan to End Mission
Andrew Lawler 189.

CLINICAL TRIALS: Facing Criticism, Industry Offers to Share Data
Jennifer Couzin 189.

INFECTIOUS DISEASE: Polio Eradication Effort Adds New Weapon to Its Armory
Leslie Roberts 190.

SOUTH ASIA TSUNAMI: U.S. Clamor Grows for Global Network of Ocean Sensors

Eli Kintisch 191.

PALEONTOLOGY: New Fossils Show Dinosaurs Weren't the Only Raptors
Erik Stokstad 192.

ITALY: Synchrotron Staff Protests Funding Cuts
Alexander Hellemans 192.

BIOMEDICINE: As the Worm Ages: Epilepsy Drugs Lengthen Nematode Life Span
Ingrid Wickelgren 193.

ACADEMIC AFFAIRS: Plan for Chiropractic School Riles Florida Faculty
Yudhijit Bhattacharjee 194.

DEVELOPMENTAL BIOLOGY: Bird Wings Really Are Like Dinosaurs' Hands
Elizabeth Pennisi 194.

STEM CELLS: California's Bold $3 Billion Initiative Hits the Ground Running
Constance Holden 195.

News Focus

DRUG SAFETY: Gaps in the Safety Net
Jennifer Couzin 196-198.

RADIATION HAZARDS: Kyrgyzstan's Race to Stabilize Buried Ponds of Uranium Waste
Richard Stone 198-200.

SOUTH ASIA TSUNAMI: Failure to Gauge the Quake Crippled the Warning Effort
Richard A. Kerr 201.

AMERICAN GEOPHYSICAL UNION MEETING: A Lively Core Turns Mercury Into an Enormous Electromagnet
Richard A. Kerr 202.

AMERICAN GEOPHYSICAL UNION MEETING: What's Going On in Saturn's E Ring?
Richard A. Kerr 202-203.

AMERICAN GEOPHYSICAL UNION MEETING: Scary Arctic Ice Loss? Blame the Wind
Richard A. Kerr 203.

AMERICAN GEOPHYSICAL UNION MEETING: Snapshots From the Meeting
Richard A. Kerr 203.

Departments

Quarterly Author Index 1-12.

Products

NEW PRODUCTS 274.

NetWatch

EXHIBITS: A Century of Relativity * DIRECTORIES: Is There a Cartographer in the House? * RESOURCES: Answering Age-Old Questions * DATABASE: Atomic Alter Egos 187

ScienceScope

Perchlorate Study Suggests Lower Risk * Is NASA Ready for Readdy? * Swansea U. Goes Deep Into Supercomputing * NIH Wants More Pioneering Women 191

Random Samples

A Clean Sweep * Monumental Makeover * Primordial Fungus * Jobs * Honors * Deaths 204

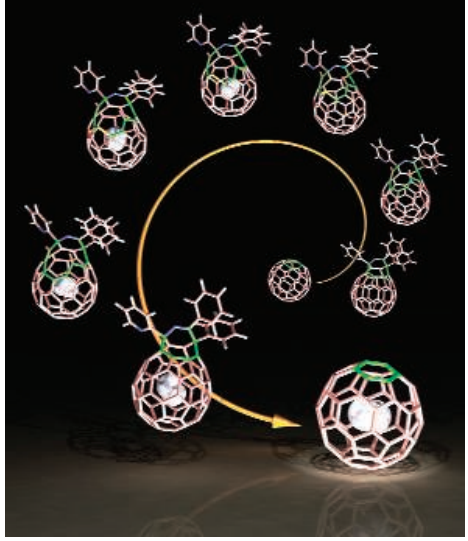
edited by Stella Hurtley and Phil Szuromi

New Tool for the TB Armory

There is an urgent need for new drugs to combat the advancing scourge of tuberculosis that is inexorably linked with the HIV epidemic. **Andries *et al.*** (p. 223, published online 9 December 2004; see the cover and Perspective by **Cole and Alzari**) have developed a lead compound from a series of recently patented diarylquinolines, known as R207910. This compound has good selectivity and potency for several mycobacterial species, including *Mycobacterium tuberculosis*, and retains activity against *M. tuberculosis* strains that are singly or multiply resistant to commonly used drugs. In contrast to other antimycobacterial drugs, R207910 targets an adenosine triphosphate synthase. R207910 enhanced mycobacterial killing in a mouse model of established infection compared with isoniazid, rifampicin, or pyrazinamide, which are used in current therapeutic regimens. It is hoped that this new drug candidate will allow the treatment of tuberculosis in as little as 2 months.

Caged Gas

Endohedral fullerenes contain guest atoms or molecules within their cages that are trapped during the synthesis of the fullerene. **Komatsu *et al.*** (p. 238) show that a C_{60} derivative that contained a large opening (a 13-membered ring) could be closed in a series of synthetic steps. In this manner, they are able to create C_{60} trapping H_2 in high yield.



CREDIT: KOMATSU *ET AL.*

Variation on a Theme

The semaphorins and their plexin-neuropilin coreceptors are established players in axon guidance. More recently, they have also been implicated in vascular development. **Gu *et al.*** (p. 265, published online 18 November 2004) report that semaphorin 3E (Sema3E) does not require neuropilin as a coreceptor in patterning the developing mouse vascular system, but instead interacts directly with the plexin-D1-expressing cells. The repulsive effect of Sema3E-bearing somites on vascular endothelial cells expressing plexin-D1 was observed in the absence of neuropilins, indicating that neuro- pilins are not, after all, obligatory semaphorin coreceptors in mammalian vasculogenesis.

Spin Switching Nanomagnets

Injecting a polarized spin current into a magnetic material can exert a torque on the magnetic moment, causing it to precess. Under the right conditions, the magnetic moment can be flipped, potentially allowing electrically controlled magnetic memories. However, details of the dynamics of this precession and switching have been lacking. **Kirivortov *et al.*** (p. 228; see the Perspective by **Covington**) now present a time-domain technique for looking at these processes. Using a magnetic nanopillar sandwich structure, they show that the precession and magnetic reversal processes are coherent processes driven by polarized spin injection.

Tuning Superatom Chemistry

Much of chemical reactivity can be understood in terms of the driving force provided by the stability of bonding arrangements that provide each atom with a closed atomic shell of electrons. For small atomic clusters, the so-called "jellium" model predicts that stable superatom clusters can form with a distinct number of valence-electrons (one such shell occurs at 40 electrons). **Bergeron *et al.*** (p. 231) build on recent work showing that $Al_{13}I^-$ forms such a superatom. They now show that Al_{13} cluster anions bearing an even number of iodine atoms show halogen-like stability, and that Al_{14} cluster anions bearing an odd number of iodine atoms show an alkaline earth–like stability. The delineation of these additional families indicates that other superatom systems may also be realized.

A Tamed Radical

Radicals, or compounds in which a single electron is missing from the valence shell of one of the atoms, act as short-lived intermediates in many chemical reactions. A series of important oxidative enzymes stabilize O-centered phenoxyl radicals by coordination to a transition metal in the active site. Whether a comparable mechanism pertains with N-centered radicals has been an open question. Now **Büttner *et al.*** (p. 235; see the Perspective by **Kaim**) have prepared a rhenium complex with a coordinated N-centered aminyl radical. The complex is stable as a solid and in a room-temperature solution. Spectroscopy, theory, and its reactivity supports a structure in which it is mainly N, not the metal center, that has lost an electron, consistent with radical stabilization by the rhenium.

Resolved Bump

Astronomers have repeatedly noted a 2175 angstrom extinction feature (or bump) in spectra of dust in the interstellar medium. The unknown source of this bump must be the most abundant species in the interstellar medium, as the feature is ubiquitous. **Bradley *et al.*** (p. 244) identified organic carbon and amorphous silica-rich material as the carriers of the 2175 angstrom bump in laboratory spectra of interplanetary dust particles that were collected in Earth's stratosphere.

An Albatross's Life

Albatrosses are well known for their extreme wide ranging foraging trips around the Southern Ocean from their colonies during the breeding season. Using leg-mounted loggers on 22 individual gray albatrosses over periods of 18 months, **Croxall *et al.*** (p. 249) provide evidence of the spectacular circumpolar migrations of albatrosses and reveal the underlying structure and strategies of these journeys. Migration strategies differed between individual birds. Some regularly circumnavigated the globe, while others either remained in the vicinity of the breeding grounds or migrated to a region in the Indian Ocean. Albatrosses are among the most endangered of all pelagic seabirds, and these data help to identify the critical habitats where protection is most required.

CONTINUED ON PAGE 179

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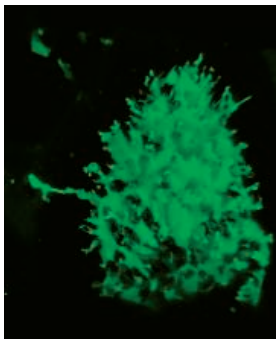
Retinoic Acid and Heart Development

Model systems such as the zebrafish heart can be used to shed light on the normal development and function of the cardiac system in vertebrates and to assist in our understanding of heart injury and disease. Retinoic acid is critical for late steps in heart development, including terminal myocardial differentiation, cardiac looping, and ventricular maturation and growth. Using zebrafish genetics and embryology, **Keegan *et al.*** (p. 247) now show that there is also an early function of retinoic acid in cardiac specification. Retinoic acid signaling is involved in selecting the number of cardiac progenitors from within a multipotential pool, and organ size is controlled by retinoic acid-mediated restriction of the early cardiac progenitor pool.

Gut Antigen Sampling and Host Defense

A complex interplay has evolved between the cells of the immune system and the mucosal barrier that interfaces with the intestinal lumen and its contents. A good example of this are the specialised antigen-presenting dendritic cells (DC) that reside below the intestinal epithelium "sampling" luminal contents via dendritic extrusions as they extend through the epithelial barrier. **Niess *et al.*** (p. 254) examined the behavior and activity of these myeloid-derived DC. The DC were regulated in the extrusion of trans-epithelial dendrites and in their phagocytic activity by the chemokine receptor CX3CR1. Loss of these activities in the absence of CX3CR1 correlated with an increase in susceptibility to *Salmonella typhimurium*, suggesting a direct link between trans-epithelial sampling of antigen by DC and immune-mediated protection of the intestinal mucosa.



CREDIT: NIESS *ET AL.*

Anticonvulsant Medications and Aging in Worms

Drugs used to treat human seizures have been found to extend the life-span of worms. **Evason *et al.*** (p. 258; see the news story by **Wickelgren**) report that adult worms exposed to three structurally similar anticonvulsant drugs had a life-span increase of nearly 50%. In addition to delaying age-related degenerative changes in worms, the drugs also increased neuromuscular activity, a behavior associated with increased life-span in the worm. The drugs may act by a common mechanism both to affect neural activity and aging, and provide potential leads as therapeutics to treat human aging.

Another Route to Stat Regulation

Stats (signal transducers and activators of transcription) efficiently carry information from cell surface cytokine receptors (which cause Stat phosphorylation) to the nucleus (where Stats work as transcriptional activators). **Yuan *et al.*** (p. 269; see the Perspective by **O'Shea *et al.***) report that Stat3 is also regulated by acetylation of a specific lysine residue. Stat3 associated with the transcriptional coactivators CBP and p300, which have histone acetyltransferase activity and can modify Stat3 in vitro. Acetylation of the key lysine residue appears to be required for dimerization of Stat3 and for transcriptional activation of genes in cells treated with the cytokine, oncostatin M. Cells expressing a mutant form of Stat3 that is not acetylated were insensitive to gene regulation and growth promotion by oncostatin M.

Testing the Strength of Hypothesis

Whether a hypothesis gets credit for predicting new data versus for when it merely accommodates old data is a controversial matter among philosophers of science. **Lipton** (p. 219) reviews several attempts to answer this question before presenting his own arguments as to how and why the ability to predict trumps the ability to accommodate existing data.

The Science of Social Diseases

The misery of life for many inhabitants of the former Soviet Union has been made shockingly plain by a grim succession of health statistics. One of the most thoroughly documented phenomena is the high death rate of young and middle-aged Russian men, linked to poor nutrition, alcoholism, cardiovascular disease, the resurgence of syphilis and tuberculosis (TB), and the spread of AIDS. This catalog of ill health is not merely a list of different ailments with separate causes, it is symptomatic of large-scale social disruption, with elements including poor education, psychological stress, rising crime and violence, high rates of unemployment, and a very unequal distribution of income among those employed.

Among these "social diseases," TB plays a leading role as the ubiquitous indicator of failing health and health services. Remarkably, Soviet health reporting systems remained intact through the turmoil of the 1990s. As a result, we know that the TB incidence rate roughly trebled in Russia between 1990 and 2000, approaching 0.1% annually by the turn of the millennium (see www.who.int/tb). A similar thing happened in all the ex-Soviet states, but not in central Europe. No one has dared to forecast how much worse the resurgent TB epidemic will get. However, as a key indicator of population health at the European Union's eastwardly mobile frontier, TB trends are being closely watched.

Against this dark background, a few bright spots are visible in the latest surveillance statistics. The 2003 data confirm that TB incidence rates in Belarus, Estonia, Latvia, Lithuania, and Russia have been falling for the past 3 to 4 years. Although this is reassuring, there will be some hesitation in accepting that the worst is over as long as the data cannot explain why. Was it because revitalized TB control programs stopped disease transmission? Or because a general recovery in population health lowered susceptibility to TB? Or did the new epidemic exhaust the supply of susceptible people to infect? Russia had actually taken steps to contain TB by 1994, when reviving treatment programs cut patient death rates. The downturn in incidence since 2000 could be the delayed effect of preventing transmission. On the other hand, the same epidemiological pattern is seen in several newly independent states, indicating that wider epidemiological processes are at work. Wealth appears to be relevant, because the fall in incidence is more conspicuous in the richer states of Soviet Europe than in the poorer countries of central Asia.

The general problem is that we often cannot know to what extent large-scale interventions contribute to observed improvements in health, because these interventions are not carried out as controlled experiments. In this context, a blueprint for reaching the UN Millennium Development Goals, to be submitted to the United Nations Secretary General on 17 January this month, will recommend a battery of specific actions to alleviate poverty. The scientific hitch is that we may never be able to prove that they succeeded, even if they are all implemented. The same difficulty faces those who will evaluate the success of the $150 million World Bank loan to Russia for TB and AIDS control and the large-scale projects now supported by the Global Fund to Fight AIDS, Tuberculosis and Malaria. The strength of the link between cause and effect will depend, in part, on how convincingly we can generalize from the original experimental proof.

Despite the complex interactions between TB and various social, biological, and economic factors, there is at least one simple message for those who are devising new health technologies. It is that without effective systems for delivery, new tools will be of little value. For instance, a new kind of drug to treat TB, such as the one reported by Andries *et al*. in this issue (see also the Perspective by Cole), would undoubtedly be a huge step forward, especially in the treatment of drug-resistant disease. But patients must want it and health services must be able to provide it. From Vilnius to Vladivostock, the typical TB sufferer is, in some combination, male, unemployed, alcoholic, HIV-positive, or in prison. The science required to make technology work in this and other social settings is tractable and could be hugely beneficial. But scientists, like patients and physicians, need incentives, and operational research remains an undervalued, and therefore underexploited, discipline.

Christopher Dye

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10.1126/science.1109116

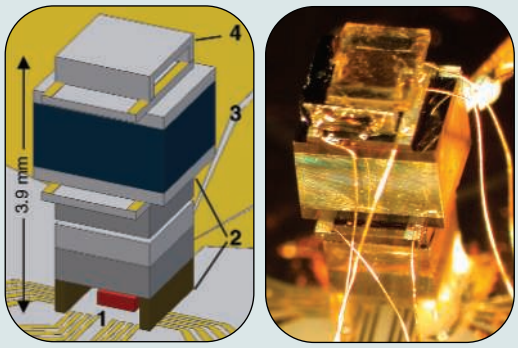
CREDIT: WHO/STB/COLORS MAGAZINE/J. MOLLISON

edited by Stella Hurtley

APPLIED PHYSICS

Chip-Scale Magnetic Measurements

The ability to measure tiny magnetic fields with good sensitivity can be found in many applications, from biological imaging to prospecting for buried treasure. However, the most sensitive magnetometers that operate in ambient conditions tend to be power-hungry, bulky, and heavy. Shrinking the size to just several millimeters and the power consumption to hundreds of milliwatts, Schwindt *et al.* have fabricated a sensitive magnetometer using microelectromechanical technology. A cloud of rubidium atoms trapped in a micromachined vapor cell is used to sense the magnetic field. The magnetic field splits the energy levels of rubidium atoms, and the extent of the splitting depends on the strength of the magnetic field. Changes in the magnetic field are then detected and tracked optically by the relative absorption changes of a laser light tuned to the split energy levels. It could be that in the not-too-distant future we could be using handheld battery-operated magnetometers. — ISO



The miniaturized magnetometer.

Appl. Phys. Lett. **85**, 6409 (2004).

NEUROSCIENCE

Making Memories

During learning, in a process termed long-term potentiation or long-term facilitation, synapses are specifically modified by a process that involves transcription. Because the synapse itself is at a distance from the neuronal cell nucleus —separated by the elongated axon or dendrite—the neuron must possess mechanisms to transmit synaptically activated second messengers and transcription factors to its nucleus. Thomson *et al.* now dissect aspects of this pathway in *Aplysia* sensory neurons and in mouse hippocampal neurons. In both cases importins (proteins involved in active nuclear import in many cell types) appear to be involved. In both types of neurons, importins were found localized along axons and dendrites and in synaptic compartments. Stimuli that triggered long-lasting facilitation in *Aplysia* triggered translocation of importin to the nucleus. Similarly, in hippocampal neurons synaptic receptor activation promoted nuclear accumulation of importin. The changes in importin distribution were not observed when only short-term synaptic changes were induced (changes that are known not to involve changes in transcription). It remains to be demonstrated which memory-related substrates may be associated with the translocating importins, but a role for the classical nuclear import pathway in generating long-lasting memories seems likely. — SMH

Neuron **44**, 997 (2004).

CLIMATE SCIENCE

Twinned Thinning

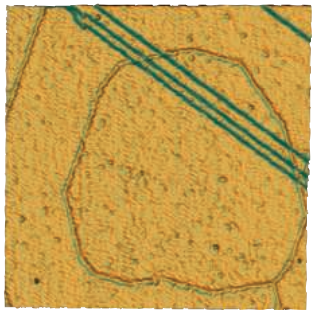
The response of the West Antarctic Ice Sheet (WAIS) to global warming is of great concern because, if it were to melt completely, it is large enough to raise sea level by approximately 7 m. Such massive melting is unlikely to occur soon; nevertheless, there is still the potential for a marked increase in the rate of sea level rise due to accelerated ice loss. The great majority of the ice mass lost presently from the WAIS flows to the sea as ice streams, of which that of Pine Island Glacier is the most important. The Pine Island Glacier, and the adjoining ice shelves of Pine Island Bay, have thinned significantly over the past 3 decades. In two related papers, the extents, causes, and effects of these changes are examined. Shepherd *et al.* use satellite data altimetry to document how ice shelves in that region have thinned, and they attribute the thinning to melting cased by the action of ocean currents that are 0.5°C warmer than freezing on average. The pattern of shelf thinning mirrors that of their grounded tributaries, suggesting that Antarctic ice is more sensitive to changing climates than previously thought. Payne *et al.* test the hypothesis that these changes are triggered by the adjoining ocean, using a numerical ice-flow model to simulate its effects on the dynamics of the Pine Island Glacier. They confirm the idea that recent increases in local ocean temperature are the cause of the observed thinning and find that the thinning of coastal ice shelves is transmitted rapidly to the grounded ice streams above, revealing a tight coupling between the ice sheet interior and surrounding ocean. — HJS

Geophys. Res. Lett. **31**, 10.1029/2004GL021106; 10.1029/2004GL021284 (2004).

CHEMISTRY

Maintaining Chains

Coupling reactions of organic molecules on surfaces can proceed at modest temperatures. McCarty and Weiss have used low-temperature scanning tunneling microscopy (STM) to observe molecules aligning into chains before such reactions can proceed. At room temperature, diiodobenzene dissociates on the atomically flat Cu(111) surface to create mobile phenylene radicals that can be pinned at defect sites. Images taken at 77 kelvin show that the phenylene species align in noncovalently bonded chains—the STM tip could be used to pull a phenylene monomer out of the chain. At higher surface coverages, a second layer of chains can align on a surface already covered with phenylene chains. Parts of the upper-level chains could be nudged to new locations



Phenylene chains hang together, even over surface steps.

CREDITS: (TOP) SCHWINDT *ET AL., APPL. PHYS. LETT.* **85**, 6409 (2004); (BOTTOM) WEISS *ET AL., J. AM. CHEM. SOC.* 10.1021/JA038930G (2004)

Published by AAAS

on the surface, where they would return to their original length by recruiting more monomer units. — PDS

J. Am. Chem. Soc. **126**, 16672 (2004).

ECOLOGY/EVOLUTION

Eats Roots or Shoots

Recently, plant ecologists have increasingly focused on the role of soil organisms in determining plant community processes. Below-ground herbivores, such as worms, tend to promote plant diversity when they feed on dominant plant species. However, van Ruijven *et al*. show that the combined effects of above- and below-ground herbivores cannot be predicted from their separate effects. Different combinations of invertebrate herbivores (nematodes and

Experimental plot.

wireworms below ground, and grasshoppers above ground) were added to experimental species-rich grassland plant communities. When added separately, the nematodes and wireworms had positive effects on diversity, whereas the grasshoppers had neutral effects. When added together, however, the combined effect on diversity was negative. The different feeding preferences of the two groups of herbivores appeared to alter the competitive interactions among the plant species within the communities, eventually producing the nonadditive effects observed. Differential distributions of above- and below-ground herbivores may well contribute to locally heterogeneous diversity levels. — AMS

Ecol. Lett. **8**, 30 (2005).

BIOTECHNOLOGY

Library Science

Bacteria are everywhere and can eat just about anything, including such unappetizing fare as petroleum sludge. Therefore, they must possess the enzymes (and the genes encoding the enzymes) that catabolize hydrocarbons. In the past, the challenge has been to identify and cultivate the desired species; advances in technology have made it feasible to bypass cultivation and to browse for specific genes (enzyme activities) in metagenome (expression) libraries. Uchiyama *et al*. take the next step in devising a method of sorting the library contents on the basis of substrate specificity and then searching for genes of interest. Their approach succeeds because bacteria rely on gene regulatory networks (and even riboswitches) that, in many cases, are induced or repressed by small molecules—either the substrate itself or chemically related compounds. Starting with a metagenome library made from petroleum-contaminated groundwater, they end up with a P450 enzyme that catalyzes hydroxylation (which makes hydrocarbons more polar and amenable to catabolism) of 4-hydroxybenzoate. — GJC

Nature Biotechnol. **23**, 88 (2005).

HIGHLIGHTED IN *SCIENCE*'S SIGNAL TRANSDUCTION KNOWLEDGE ENVIRONMENT

Science's stke www.stke.org

Specificity Through Degradation

Yeast use partially overlapping kinase modules to specify discrete cellular responses. For example, the upstream kinases in the mitogen-activated protein kinase (MAPK) cascade, Ste11 and Ste7, are both activated during mating response signaling and during filamentous growth signaling. The MAPK Kss1 then triggers the filamentous growth transcriptional cascade and the MAPK Fus3 triggers the mating response genes. In the absence of Fus3, pheromone signaling stimulates Kss1 and filamentous growth gene expression, suggesting that Fus3 has a role in suppressing filamentous growth responses during pheromone signaling. Chou *et al*. and Bao *et al*. now report that Fus3 triggers the degradation of a transcription factor required for filamentous growth, Tec1, to maintain signaling specificity through the shared MAPK pathways. The abundance of Tec1 decreased after mating stimulated by pheromone and this destabilization required Fus3 but not Kss1. Tec1 Thr273 was phosphorylated by Fus3. Degradation was mediated by a SCF ubiquitin ligase complex. Thus, selective degradation of a transcriptional regulator represents a mechanism for generating specificity during intracellular signaling. — NG

Cell **119**, 981 (2004).

CREDITS: J. VAN RUIJVEN, IMPERIAL COLLEGE, LONDON

Published by AAAS

NetWatch

edited by Mitch Leslie

EXHIBITS

A Century of Relativity

In 1905, 26-year-old patent clerk Albert Einstein showed that light consisted of particles, launched his theory of special relativity, and crushed the remaining doubts about the existence of atoms. Not too shabby for a part-time physicist whose parents had once fretted that he was dumb. Kick off the 100th anniversary of Einstein's "miraculous year" by visiting a newly revised exhibit on him from the American Institute of Physics (AIP). Along with a 100-page tour of his life and work, the site now holds essays by leading Einstein scholars, who explore topics such as the genesis of special relativity. Other new features include a revamped bibliography and a chronology of Einstein's achievements in 1905.

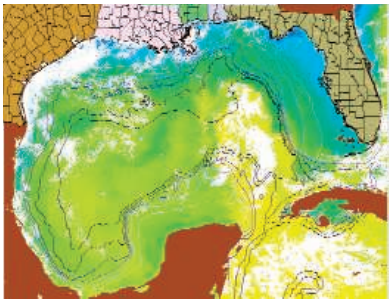
The Einstein exhibit is one of 10 online displays from AIP's Center for History of Physics, covering subjects from nuclear researcher Werner Heisenberg to the history of the transistor. You can also browse more than 25,000 portraits, snapshots, and other images of physicists from the center's visual archive.

www.aip.org/history

DIRECTORIES

Is There a Cartographer in the House?

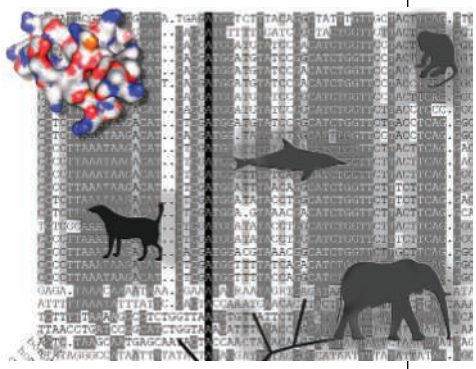
Looking for maps that delineate recent outbreaks of potentially dangerous algae? How about county-by-county charts of infant mortality in the southern United States? At the portal Geodata.gov, you can quickly find loads of mappable data mainly from the federal government. Whether it's the locations of wetlands or crop-growing conditions around the world, the site provides a brief description of the data set and a link to its home. Many of the original sites offer their own mapping features, but Geodata.gov allows you to combine data sets from different sources. In this map showing the Gulf of Mexico in December 2004, the red dots off Florida indicate toxic algae.



www.geodata.gov/gos

RESOURCES

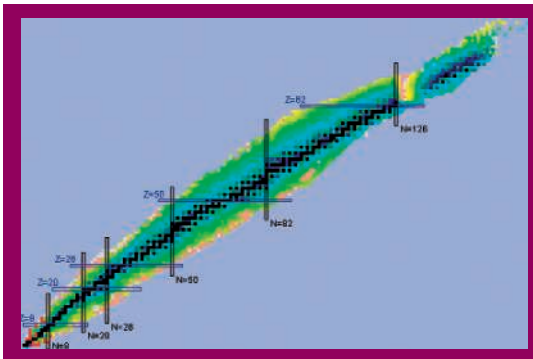
Answering Age-Old Questions



No mouse has survived longer than 5 years. A lucky lion might reach 30, and the oldest person on record was still enjoying the occasional glass of port until her death at age 122. How fast various organisms age boils down to differences in their genes. That's the premise of the 3-year-old Human Ageing Genomic Resources site, a collection of databases for teasing out genetic influences on aging.

The site's centerpiece is a database that characterizes more than 200 genes linked—tenuously or strongly—to human aging. Each gene's file describes its protein product's function and relevance to aging, lists other proteins it mingles with, identifies corresponding genes in model organisms, and more. For researchers interested in comparative aging, another database tallies demographic and physiological variables such as record life span, basal metabolic rate, and maturation time for more than 2000 species. Project leader João Pedro de Magalhães, a Harvard postdoc, also runs the parent site senescence.info, which brims with background information. You can compare theories for why organisms grow old or read about purported antiaging treatments. Don't celebrate just yet—none of them has been shown to work.

genomics.senescence.info



DATABASE

Atomic Alter Egos

Breaking up is easy to do for unstable isotopes such as uranium-235 and nitrogen-17. Everyone from nuclear engineers to health physicists can corral basic data about these fleeting isotopes and their more stable counterparts at NuDat from Brookhaven National Laboratory in Upton, New York. For nearly 3000 isotopes, the site records properties such as spin-parity, half-life, mass, and type of radioactive decay. To learn more about a particular breakdown, try the Decay Radiation function, which supplies values such as energy release and radiation dose. The chart above plots the different isotopes by their number of neutrons and protons.

www.nndc.bnl.gov

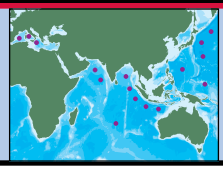
Send site suggestions to netwatch@aaas.org. Archive: www.sciencemag.org/netwatch

CREDITS (TOP TO BOTTOM): LIBRARY OF CONGRESS/AIP; JOÃO PEDRO DE MAGALHÃES; NATIONAL NUCLEAR DATA CENTER/BNL; NOAA

Published by AAAS

This Week

PAGE 190
"More wallop per punch"



191 & 201
After the tsunami

ATMOSPHERIC SCIENCES

NOAA Loses Funding to Gather Long-Term Climate Data

Congress has eliminated funding for a fledgling network of 110 observation stations intended to provide a definitive, long-term climate record for the United States.

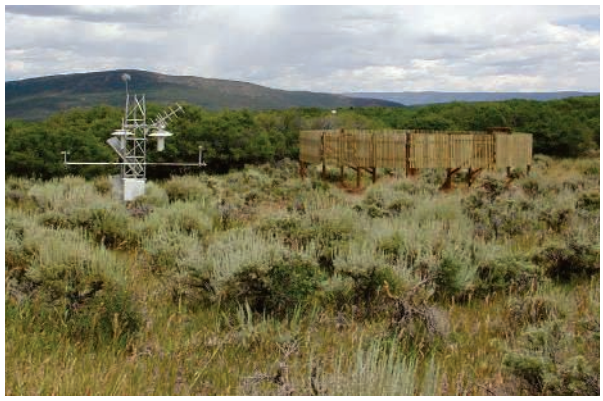
The surprise assault on the Climate Reference Network (CRN) was buried in the 3000-page omnibus spending package for 2005 signed last month by President George W. Bush (*Science*, 3 December 2004, p. 1662). Legislators also took a bite out of a long-established atmospheric monitoring network that includes the historic time sequence of increasing carbon dioxide levels measured at Hawaii's Mauna Loa. Both networks are key pillars in a much-touted international "system of systems" for earth observation that the Bush Administration has called essential for resolving uncertainties in the connection between greenhouse gas emissions and climate change (*Science*, 20 August 2004, p. 1096). While federal officials say they plan to "limp along" this year and hope for better news in 2006, some scientists worry that the cuts signal a lack of political support for filling those gaps.

"[CRN] ties everything together," says Richard Hallgren, former director of the National Weather Service and executive director emeritus of the American Meteorological Society. "Eliminating it would be an absolute disaster."

The excision of CRN's $3 million budget is part of a $10.6 million cut in the $24.3 million climate observations and services program, which supports a far-flung monitoring system operated by the National Oceanic and Atmospheric Administration (NOAA). The reference network was part of the president's 2005 request for NOAA and was funded in separate bills that had moved through the House and Senate. But "it disappeared" after conferees completed work on the massive bill that bankrolled dozens of federal agencies, notes program head David Goodrich.

CRN is meant to provide a 50-year climate record—including solar radiation, wind speed, and relative humidity—that is of much higher quality than existing temperature and precipitation records from weather stations. The weather stations are often staffed by volunteers, and the data are undermined by changing urban conditions, poor maintenance, and other variables. In contrast, CRN will rely on state-of-the-art equipment located in protected areas such as national parks and inspected regularly. "This network," says Thomas Karl, its moving force as director of NOAA's National Climate Data Center in Asheville, North Carolina, "will eliminate the adjustments and corrections that we've had to make in the data" that have spawned so much debate about recent U.S. climate trends.

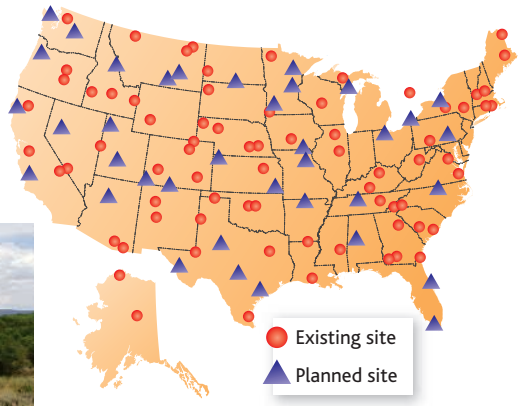
But this year's budget squeeze, he says, raises questions about the viability of the network, begun in 2001 and with 56 stations now operating. For starters, the cuts will force 16 new stations scheduled to be commissioned this year into "hibernation." It also means no money for some 20 technicians who crisscross the country to tend the equipment. Karl has siphoned off $1.5 million from other programs to keep on a skeletal maintenance crew. But he's worried that the hibernating stations could become degraded without proper maintenance and that further delays could trigger a clause in its site leases that requires NOAA to dismantle the entire system if the stations are not in use.



Stationary system. NOAA's plans for a nationwide climate network, like this station in Gunnison National Park in Colorado, have taken a hit from Congress.

Also at risk are the five observatories operated by NOAA's Climate Monitoring and Diagnostics Laboratory (CMDL) in Boulder, Colorado. These sites, from Alaska to the South Pole, measure levels of carbon dioxide, carbon monoxide, methane, halogenated compounds, ozone, aerosols, and other atmospheric constituents. The data help researchers build better climate models.

A $2.5 million budget cut means that the observatories will be serviced less often, and several contractors will be given the boot, says CMDL Director David Hofmann. That will increase the burden on an aging system that, among other achievements, includes a Hawaiian project begun by Charles Keeling in 1958 that first alerted the world to a steady rise in $C0_2$ levels. "The road is barely passable now," Hofmann says about the 180-kilometer roundtrip to the Mauna Loa summit. "At some point we won't even be able to make it up there."



CREDITS: NCDC/NOAA

Beyond the loss of data from individual monitoring stations, the cuts jeopardize the Bush Administration's Global Earth Observing System of Systems (GEOSS), a planned linking of existing networks to paint a comprehensive, real-time picture of what's happening to the planet. "It raises the question of whether the nation is willing to support a sustained, long-term effort to do the best possible job of monitoring our climate," says Kenneth Kunkel of the Illinois State Water Survey, who chairs CRN's ad hoc science working group.

To Kevin Trenberth, head of the climate analysis section at the National Center for Atmospheric Research in Boulder, the message from legislators is even bleaker. "It's almost as if some people don't want to know how the climate is changing," he says. "Maybe they prefer uncertainty, so that they can avoid taking action."

—JEFFREY MERVIS

Published by AAAS

195
Regenerating controversy

196
Back into the bottle?

198
A sea of Soviet waste

RAINFALL MONITORING

Report Bucks NASA's Plan to End Mission

The forecast for an aging NASA spacecraft that keeps tabs on tropical rainfall turned stormy last week. A National Academies' panel released an interim report urging the space agency to keep the satellite flying at least through the end of the year. But NASA officials insist they may have to shut it down as early as this summer, before the academy can finish its study.

Both climate researchers and weather forecasters are eager to continue gathering data from the joint U.S.-Japanese Tropical Rainfall Monitoring Mission (TRMM) launched in 1997. They argue that the instruments could continue beaming back data for another 6 years. But NASA says that unless the National Oceanic and Atmospheric Administration (NOAA) agrees to take over operations, the constraints of time, money, and safety will force it to shut off instruments.

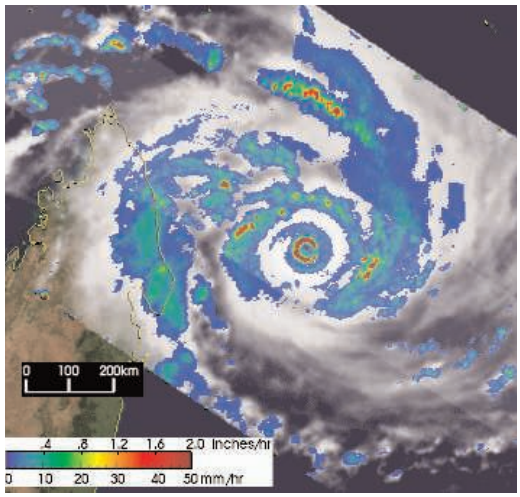
NASA requested the study after scientists and members of Congress criticized agency plans to halt operations last summer (*Science*, 13 August 2004, p. 927). The academy panel, chaired by Eugene Rasmusson of the University of Maryland, College Park, "strongly recommends continued operation of TRMM," at least through the end of 2005. The panel notes that TRMM's precipitation radar and microwave imager in particular provide a "powerful" set of data points for long-term understanding of rainfall patterns as well as near-term observation of hurricanes. It says TRMM also complements NOAA's polar weather satellites, which fly in a different orbit. "The instruments are in excellent shape," says project scientist Robert Adler of NASA's Goddard Space Flight Center in Greenbelt, Maryland.

But managers at NASA headquarters say they can't keep TRMM flying. "The real dilemma is physics, not money," says one NASA official. The longer the satellite remains in orbit, the greater the risk that it cannot be sent into a controlled reentry above the Pacific Ocean and the more resources—personnel to monitor the satellite—will be needed. So while it would cost $4 million a year to continue operating TRMM, the reentry effort could take years and cost as much as $16 million. Meanwhile, NASA wants to spend every available penny to build a Global Precipitation Mission that would provide broader coverage starting later in the decade.

NASA deputy science chief Ghassem Asrar said that, although TRMM has yielded "significant scientific data," the agency must remain "vigilant" to ensure a controlled reentry. And that could mean shutting off the instruments as early as summer. "The sooner we prepare for deorbit, the better," he adds. TRMM advocates say an uncontrolled reentry does not pose a significant risk, however, citing a 2002 finding by NASA's own safety directorate. "The community is going to have to speak out," says Adler.

But wanting the data isn't enough. Somebody—NOAA, Congress, the White House, or Japan—must also come up with the money and persuade reluctant NASA managers to keep TRMM on the job.

—ANDREW LAWLER



Rainmaker. Cyclone Gafilo pounds Madagascar last winter.

CREDIT: HAL PIERCE, SSAI/NASA GSFC

CLINICAL TRIALS

Facing Criticism, Industry Offers to Share Data

Five trade groups representing pharmaceutical companies worldwide are urging members to release more information about clinical trials. However, some see the proposals as a way to stay ahead of legislation that could compel the release of such information.

The companies have been under pressure since revelations that they kept trial data for antidepressants and other drugs secret. Congress failed to act last year on calls for a mandatory clinical trials registry, with penalties for noncompliance, but those bills are expected to reappear. The co-sponsor of one such bill, Representative Henry Waxman (D–CA), said last week that "nothing" in the industry's announcements "is going to dissuade me" from pursuing legislation. But the Pharmaceutical Research and Manufacturers of America (PhRMA), a Washington, D.C.–based trade group, says it would prefer for Congress to wait and "see if the voluntary efforts are going to work," says spokesperson Jeff Trewitt.

Voluntary registries in the past have included only a fraction of ongoing and completed trials. Seven of the nearly 100 members of the Association of the British Pharmaceutical Industry (ABPI) have participated in its registry, launched in May 2003. A 2003 study of U.S. cancer trials found that fewer than half of those sponsored by industry appeared on the government Web site (clinicaltrials.gov).

The U.K.'s ABPI is pinning its hopes on the World Health Organization's efforts to establish a global trials database by July; it will recommend that members post trials and results there. The new PhRMA plan recommends adding trials for all ailments to clinicaltrials.gov.

Other groups behind the effort include the European Federation of Pharmaceutical Industries and Associations, the International Federation of Pharmaceutical Manufacturers & Associations, and the Japan Pharmaceutical Manufacturers Association. They recommend the release of "all clinical trials to determine a medicine's therapeutic benefit," says Richard Ley, an ABPI spokesperson.

Critics such as Drummond Rennie, deputy editor of the *Journal of the American Medical Association*, aren't optimistic. "Marketing forces and self-interest … are going to win out every time over the ethics of doing the right thing," he says.

—JENNIFER COUZIN

Published by AAAS

INFECTIOUS DISEASE

Polio Eradication Effort Adds New Weapon to Its Armory

With success still frustratingly elusive, the leaders of the global program to eradicate poliovirus are reintroducing an old tool to fight the disease: an oral polio vaccine designed specifically to protect against the most pervasive strain of poliovirus, known as type 1. The only vaccine used in the 16-year eradication campaign targets three strains of the virus. The new monovalent oral polio vaccine (mOPV)—a version of which was used extensively before the adoption of trivalent OPV in the 1960s—offers "more wallop per punch," says Bruce Aylward, who coordinates the program from the World Health Organization (WHO).

It is not a silver bullet, caution officials at WHO and the U.S. Centers for Disease Control and Prevention. Program officials also stress that mOPV will augment, not replace, the well-honed strategy of immunizing every child under age 5 where polio remains a threat with several doses of trivalent OPV each year. But if mOPV works as hoped, "it may be what it takes to tip the scale," says David Heymann, who heads WHO's eradication effort.

Ripe environment. Poliovirus persists in the slums of India and Egypt.

The project, which began in November, is on an accelerated track. Sanofi Pasteur in Lyon, France, and Delhi-based Panacea Biotec have promised to deliver 200 million doses this spring. WHO officials say this could well be the fastest a vaccine has been produced and approved. The agency actually wanted the vaccine even sooner, says Francois Bompart, vice president of medical affairs for Sanofi, but the company simply could not retool production from trivalent OPV fast enough. Still, if the vaccine is ready by May, as planned, the partners should be able to deliver two rounds in Egypt and parts of India before the beginning of the high season in July to September, when viral transmission peaks. The mOPV plan, to be announced by the end of January, offers another key benefit: It will give officials a leg up on testing a key component of the vaccine stockpile needed to deal with emergency outbreaks once eradication is achieved.

The use of mOPV is designed to root out the virus in areas where it is most entrenched—typically, overcrowded slums with abysmal sanitation and booming birthrates, like greater Cairo and parts of western Uttar Pradesh, Bihar, and Mumbai in India. Despite dramatic increases in the number of national immunization days last year and the percentage of children reached in each one, viral transmission still persists in these areas, notes Hamid Jafari, who directs the global immunization division at CDC. Meanwhile, the epidemiology of the disease has shifted, says Roland Sutter of CDC. The program has successfully cleared the world of type 2 poliovirus, he says, and type 3 is "just hanging on by its teeth." In Mumbai and all of Egypt, for instance, type 3 has not been detected since October and December 2000. That opens the door for the reintroduction of mOPV against type 1 poliovirus.

Seroconversion After One Dose of mOPV, by Climate

Country category and mOPV type	Rate of seroconversion, median % (range)
TEMPERATE*	
1	95 (90-100)
2	98 (83-100)
3	94 (70-100)
NONTEMPERATE†	
1	81 (53-89)
2	89 (77-93)
3	72 (52-80)

* China, Czechoslovakia, Denmark, Estonia, Hungary, The Netherlands, United States, Union of Soviet Socialist Republics.

† Brazil, India, Mexico, Singapore, Uganda.

Since the early 1970s, polio experts have known that trivalent OPV simply isn't as effective in hot tropical climes, requiring perhaps five to eight doses to confer immunity instead of the standard three (*Science*, 26 March 2004, p. 1960). In Egypt and parts of India, especially, conditions are "very, very ripe for the virus," says Jafari. Although Egypt recorded just one case of paralytic polio in 2004, environmental samples collected from open sewers show that the type 1 poliovirus is well established in the ecosystem. The same is true in parts of India; Mumbai, for instance, reported just one case of paralytic polio in 2004, but 84 environmental samples tested positive. "So the question is, do we keep pounding away, or do we get some sharper edge to our tool?" asks Jafari. He suspects that edge will come from a new version of mOPV.

Past experience with mOPV has demonstrated that it is much more potent in prompting an immune response. Data from five tropical countries showed that just one dose of mOPV type 1 conferred immunity in 81% of those vaccinated, says Sutter. By contrast, the seroconversion rate for one dose of trivalent OPV in tropical countries is roughly 30% to 40%. The benefit occurs because the live attenuated vaccine virus, which replicates in the gut, doesn't have to compete with the other two virus types for cells susceptible to infection.

MOPV also has a long safety record, notes Bompart. But because no company has produced it in years, and it is no longer licensed, the vaccine must be reviewed as a new product. Regulatory agencies in Egypt and India have agreed to expedite the review based on historical data, while also requiring new clinical trials and postmarketing studies.

Sanofi is manufacturing 50 million doses for Egypt, and Panacea is ready to produce up to 150 million for India for introduction in May. Although all children under age 5 in the target areas will receive mOPV, the partners expect the biggest payoff to come from vaccinating very young children with low or little immunity, who are most likely to transmit the disease: "We really do need to get the youngest ones immunized as quickly as possible," says Sutter. "MOPV will help us do it faster."

At this stage, cautions Aylward, the benefits are theoretical. And even if mOPV does boost immunity as expected, says Bompart, it is not clear that it will make a "real world" difference in terms of stopping transmission. One concern is that the promise of a more effective vaccine will divert attention from the need to reach every single child with multiple doses of trivalent OPV, which must continue, says Aylward.

Even so, the idea is gaining steam. Polio expert Paul Fine of the London School of Hygiene and Tropical Medicine says the plan makes "good sense" scientifically and also shows that the program has an "open-mindedness" toward new tactics and vaccines, which may be needed to finish the job.

—LESLIE ROBERTS

CREDITS (LEFT TO RIGHT): L. ROBERTS; ADAPTED FROM V. M. CACERES AND R. W. SUTTER, *CLINICAL INFECTIOUS DISEASES* **33**, 531 (2001, UNIVERSITY OF CHICAGO PRESS); (INSET PHOTO) WHO

Published by AAAS

SOUTH ASIA TSUNAMI

U.S. Clamor Grows for Global Network of Ocean Sensors

An oft-ignored plea to the U.S. government to improve a federally funded tsunami warning system is falling on more receptive ears in the wake of the tragedy in South Asia.

Scientists at the National Oceanic and Atmospheric Administration (NOAA), which runs a six-buoy network of pressure sensors in the Pacific Ocean, have seen previous efforts to expand the network rejected on fiscal grounds. But last month's earthquake and tsunami, which have claimed at least 150,000 lives, have changed the terms of the debate. "If there was a window of opportunity, this would be it," says Jay Wilson, an earthquake and tsunami coordinator for Oregon's office of emergency management.

Completed in 2001, the Deep Ocean Assessment and Reporting of Tsunamis (DART) network is made up of six sensors tethered to the ocean floor that can detect tsunamis as small as 1 centimeter, relaying data instantly via satellite from buoys to tsunami warning centers in Alaska, Washington state, and Hawaii. Two detectors currently sit off the coasts of Washington and Oregon, three operate near Alaska, and one sits about 1000 km south of the equator. NOAA scientists believe that about 20 detectors could provide adequate coverage for coastal warnings around the Pacific, and 50 would provide the basis of a global system. But NOAA's budget makes no provision for any expansion of the current network.

Enlarging the DART system is "one of the things we're looking at," says a spokesperson for the White House Office of Science and Technology Policy, which convened a meeting last week of several federal agencies that support related research. Last week, in separate teleconferences with Senate staff and House members and staff, Eddie Bernard, the director of NOAA's Pacific Marine Environmental Laboratory in Seattle, Washington, ended a presentation on previous tsunami studies with a proposal for a 53-detector global DART array (see map).

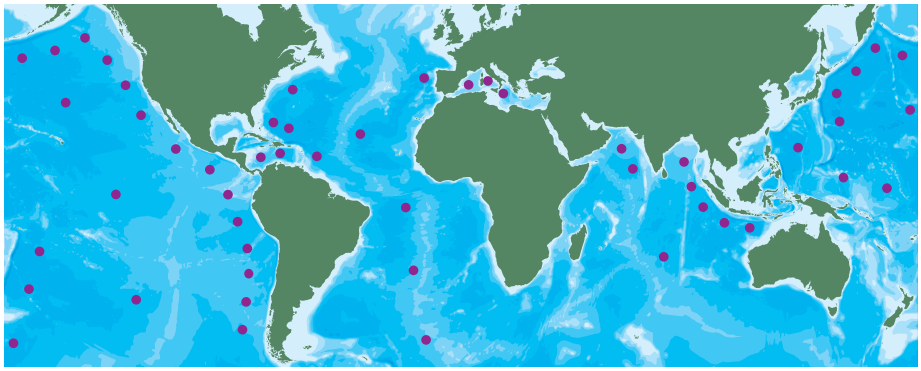
"The grand scheme is a global approach," says NOAA oceanographer Frank Gonzalez, who leads the agency's tsunami research program. The House Science Committee plans a hearing this winter on improved tsunami warning systems for U.S. and international shores, according to a staffer.

Some legislators aren't waiting. For example, on 6 January, Senator Joe Lieberman (D–CT) proposed that the United States, along with "cooperating nations," expand the DART network.

However, even a global system would have limitations, notes U.S. Geological Survey (USGS) seismologist David Oppenheimer, pointing to a 1700 earthquake on the Cascadia subduction zone that sent giant tsunami waves crashing into the Pacific coast of North America in minutes. In such a situation, he says, "the buoys aren't going to save anybody; there's just so little time."

In the meantime, science agencies are already helping researchers eager to work at the affected sites. The National Science Foundation is funding several teams studying the tsunami's behavior along coastlines in Sri Lanka and India. The foundation has also described to White House officials how it could expand its portfolio in telemetry and sensing to improve the Global Seismographic Network, which it funds. And altimetry data from the joint U.S./French JASON-1 satellite have provided scientists a rare glimpse into the tsunami's birth. "The satellite just happened to be passing over as the tsunami was taking shape," says NASA spokesperson Gretchen Cook-Anderson.

–ELI KINTISCH



Deep blue. NOAA oceanographer Eddie Bernard told lawmakers last week how an expanded network of tsunami detectors could be deployed.

CREDIT: (TOP TO BOTTOM) ADAPTED FROM PMEL; NASA

ScienceScope

Perchlorate Study Suggests Lower Risk

A new report on the health effects of the chemical perchlorate is stirring the waters on this controversial pollutant from rocket fuel. The National Academy of Sciences (NAS) study, released this week, found that the Environmental Protection Agency's (EPA's) 2002 draft risk assessment of safe daily oral intake was roughly 20 times too stringent—a figure that's prompting dissent on both ends of the spectrum.

The biggest worry about perchlorate is the harm it may cause fetuses and infants, by preventing the thyroid gland from making hormones crucial for brain development. After reviewing the existing evidence, the NAS panel determined that 0.0007 mg per kilogram of body weight is a safe level for oral intake. But environmentalists say that the study on which the panel relied most heavily only looked at adults and that infants are more sensitive to the chemical. Conversely, industry officials argue that perchlorate is safe in drinking water at even higher levels.

Both EPA and the states will likely consider the NAS report when finalizing drinking-water standards in the coming years, says endocrinologist Thomas Zoeller of the University of Massachusetts, Amherst. Another big unknown is how much perchlorate infants ingest through food and milk.

–ERIK STOKSTAD

Is NASA Ready for Readdy?

With NASA Administrator Sean O'Keefe planning to leave the space agency 1 February, the White House is scrambling to come up with a replacement. The current leading candidate is Bill Readdy, the agency's space flight chief and a former shuttle astronaut who has been with the agency since 1986. But some NASA and industry officials consider him too wedded to the space shuttle program and not enthusiastic enough about President George W. Bush's exploration vision, announced 1 year ago (*Science*, 23 January 2004, p. 444). If nominated, Readdy will also have to answer questions about the 2003 Columbia tragedy.

–ANDREW LAWLER

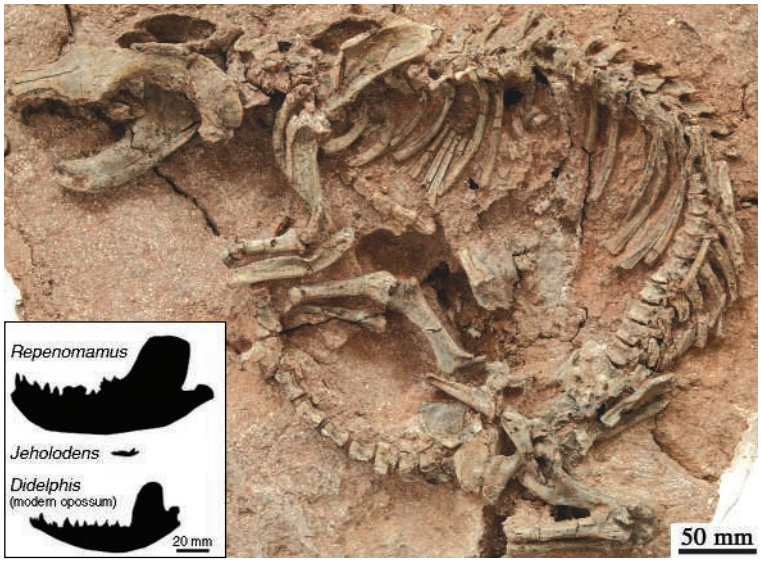
Published by AAAS

PALEONTOLOGY

New Fossils Show Dinosaurs Weren't the Only Raptors

The Mesozoic era is called the "Age of Dinosaurs" for good reason. For 185 million years, they diversified with ferocious gusto, evolving into a panoply of predators and prey that fill the record books for size and shapes. Mammals, meanwhile, were nocturnal, shrewlike nobodies that snatched insects and stole the occasional egg. Only after dinosaurs went extinct 65 million years ago could mammals escape from the shadows and begin to thrive. Or so the story goes.

In this week's issue of *Nature*, Chinese paleontologists describe the largest Mesozoic mammal skeleton ever found, more than a meter long. And this furry Goliath wasn't content just to eat bugs: A smaller relative was discovered nearby with the bones of a baby dinosaur in its stomach. "This thing was probably hunting and eating relatively large-sized dinosaurs," says Guillermo Rougier of the University of Louisville, Kentucky. "It forces us to think about [Mesozoic] mammals as a fully diversified group, not just in their typical role of insectivores."



Big guy. *Repenomamus giganticus* was much larger than other Mesozoic mammals, such as the typical shrew-sized insectivore *Jeholodens*.

The new fossils, each about 130 million years old, come from the famous fossil beds of Liaoning Province in northeastern China. Paleontologists had already discovered skulls of the smaller animal, called *Repenomamus robustus* (*Science*, 12 October 2001, p. 357), but could get only a vague estimate of its body size. Now the same team has found a fairly complete specimen of an adult. Squat, with powerful legs, it probably weighed about 4 to 6 kilograms. "We would say it looked something like a Tasmanian devil," says team member Yaoming Hu, a graduate student at the City University of New York. Collaborators include his adviser Jin Meng of the American Museum of Natural History in New York City and colleagues at the Institute of Vertebrate Paleontology and Paleoanthropology in Beijing.

While removing rock from the specimen, preparators made a rare discovery: teeth and bones strewn about inside the ribcage, in the likely position of the animal's stomach. The jumble included the remains of a herbivorous dinosaur hatchling, a 14-centimeter-long *Psittacosaurus*. One leg appears mostly intact, suggesting that the mammal dismembered and wolfed down its food. Given the large, sharp teeth and powerful lower jaw, the team suspects that *Repenomamus* was a predator, but Hu acknowledges it's hard to tell scavengers from hunters.

R. robustus wasn't the only mammal that dinosaurs had to worry about. Another skeleton, better preserved, was even larger. Named *Repenomamus giganticus*, it was 1 meter long and weighed roughly 12 to 14 kg, as much as a modern coyote. "It was probably competing with carnivorous dinosaurs for food and territory," Hu says.

And that raises interesting questions, notes Anne Weil of Duke University in Durham, North Carolina. "What these finds really allow us to do—at least speculatively—is ask how mammals might have influenced dinosaur evolution," she says. In other words, Mesozoic mammals may have cast a shadow of their own.

–ERIK STOKSTAD

ITALY

Synchrotron Staff Protests Funding Cuts

NAPLES, ITALY—The 250 employees of Sincrotrone Trieste, which operates Elettra, Italy's large synchrotron light source, put down their tools for a day this week to protest government funding cuts that triggered a financial crisis. After it lost half its income in 2002, the facility took out bank loans, which it assumed that the government would pay off. Staff and users now fear that if the government does not come to its rescue, the synchrotron may have to be mothballed. "The laboratory is suffering. If something breaks down, we cannot repair it," says Silvia Di Fonzo, a physicist at Sincrotrone Trieste and a labor union representative who helped organize the strike.

Like other synchrotrons, Elettra speeds electrons around a particle accelerator to produce x-rays that researchers use as probes in a wide variety of fields. Commissioned in 1993, Elettra hosts 840 users per year from across Europe and developing countries. But in 2002 the government drastically cut some research institution budgets, including one that supports Elettra. As a result, Elettra lost 50% of its $33 million yearly operating budget, although it retained the half that comes directly from government.

On borrowed time. The Elettra synchrotron.

According to Alfonso Franciosi, CEO of Sincrotrone Trieste, the government encouraged the company to take out bank loans to cover the shortfall. "The lab operated for 3 years with loans from local banks, and the debts are now adding up to [$20 million]," says Franciosi. The government has repeatedly promised to restore Elettra's missing $18 million per year starting in 2005, he adds. But many were alarmed to see that Elettra is not included in the 2005 government budget, which was approved last month. Elettra officials are hoping that new funding will be included in a decree on national competitiveness that the government will issue at the end of January.

Guido Possa, Italy's deputy research minister, says the trouble is that Sincrotrone Trieste was set up as a private company, making it hard for the government to fund it directly. "The problem is when you have to manage public money, you have to follow certain rules."

–ALEXANDER HELLEMANS

Alexander Hellemans is a writer in Naples, Italy.

CREDITS (TOP TO BOTTOM): ADAPTED FROM YAOMING HU *ET AL.*, *NATURE* **433**, 149 (2005); COURTESY OF SINCROTRONE TRIESTE SCPA

BIOMEDICINE

As the Worm Ages: Epilepsy Drugs Lengthen Nematode Life Span

Although pharmacists have proven medications for ailments as varied as migraines and bacterial infections, they have little to offer in the fight against aging other than unproven remedies. But new evidence suggests that the right prescription for longevity may already be hidden behind the pharmacy counter.

Geneticist Kerry Kornfeld and his colleagues at Washington University in St. Louis, Missouri, report on page 258 of this issue that a class of antiseizure drugs markedly extends the life span of the roundworm *Caenorhabditis elegans*. The scientists screened 19 classes of medications prescribed for other uses for potential longevity effects. "These compounds are approved for human use, so they have [molecular] targets in humans," says Kornfeld, although he cautions that there is no evidence yet that the anticonvulsants he tested slow aging in people.

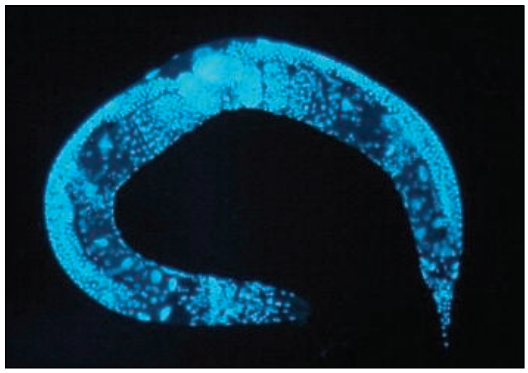
Because these drugs act on the neuromuscular systems of both humans and worms, the finding also hints at a direct link between the neuromuscular system and the aging process, says geneticist Catherine Wolkow of the National Institute on Aging in Baltimore, Maryland. Furthermore, the data indicate that although the drugs' mechanisms of action partly involve molecular pathways already known to govern aging, those pathways tell less than the whole story. "The work opens up the possibility that there may be new targets not yet explored that affect aging and neuromuscular function," says Wolkow. "That's a pretty important finding."

With a life span of a few weeks in the lab, *C. elegans* is a favorite subject for longevity studies. Since the early 1990s, researchers have linked mutations in dozens of worm genes to extensions of the creature's lives. Given all the drugs on the market, Kornfeld speculated that at least one of them was likely to retard aging or promote longevity by affecting those gene targets.

So about 4 years ago, Kornfeld's graduate student Kimberley Evason began exposing separate groups of 50 worms to various drugs, from diuretics to steroids, at three different dosages. Most of the compounds the worms ate off their petri dishes had toxic effects. After 8 months of negative results, Evason tested the anticonvulsant ethosuximide (Zarontin). A moderate dose, she found, extended the worm's median life span from 16.7 days to 19.6 days, a 17% increase. Lower doses had a lesser effect, and higher doses were toxic.

Evason then discovered that two related anticonvulsants also lengthened worms' lives, one of them by as much as 47%. By contrast, a chemically related compound that does not have antiseizure activity had no similar effect. That is "nice evidence" that the compounds' ability to extend life span is related to their effectiveness as anticonvulsants, says geneticist Javier Apfeld of Elixir Pharmaceuticals in Cambridge, Massachusetts.

The drugs are thought to control seizures in people by acting on certain neuronal calcium channels. Exactly how the drugs extend life span in worms is unknown, although they seem to stimulate the nematode neuromuscular system. Kornfeld's team discovered that the drugs affect two types of neurons: those that govern egg laying, leading to earlier release of eggs, and those that control body movement, making the worms hyperactive.



Staying alive. Anticonvulsant drugs promote longevity in roundworms like this one.

Unlike many of the genetic mutations that affect worm longevity, the drugs don't act primarily through the worm's insulin-like signaling system, the St. Louis group revealed. For example, treatment with two of the anticonvulsants still lengthened the lives of worms with life-curbing mutations in an insulin-pathway gene. "We think the nervous system effects are more complicated than simply regulating insulin signaling," Kornfeld says.

The next step is to test whether the drugs have any antiaging effects on higher organisms, such as flies and mice. "The nervous system might have a central function in coordinating the progress of an animal through its life stages, leading ultimately to degeneration," Kornfeld speculates. Still, he adds, "it's very early days for understanding the connection between neural function and aging."

–INGRID WICKELGREN

CREDIT: THE NATIONAL HUMAN GENOME RESEARCH INSTITUTE

ScienceScope

Swansea U. Goes Deep Into Supercomputing

CAMBRIDGE, U.K.—"Deep computing" is the glittering phrase IBM holds out to universities that join it in R&D projects—the latest being Swansea University in Wales. The school and IBM are jointly investing in a 1.7- to 2.7-teraflops supercomputer from the Armonk, New York, company, along with software and training for high-tech medical studies.

Dubbed "Blue C," the computer is the ballast in Swansea's planned $100 million Institute of Life Sciences (ILS). Officials expect ILS to focus on visualization, medical nanotechnology, and personalized medicine. The Welsh Assembly has added about $35 million to $6 million from private sources in hopes that the institute will generate what Wales's economic development minister Andrew Davies calls "massive economic wealth." The rest of the $100 million will be raised piecemeal.

IBM representative David White says the company's goal is to whet the appetites of top researchers for its products. It has previously partnered with the Karolinska Institute in Stockholm, the Mayo Clinic in Rochester, Minnesota, and the University of Cambridge, U.K.'s Cancer Research Center.

–ELIOT MARSHALL

NIH Wants More Pioneering Women

The National Institutes of Health (NIH) is seeking more women to apply for—and judge—its new no-strings-attached awards to innovative researchers.

The Pioneer Award, created last year as part of NIH Director Elias Zerhouni's "Roadmap" initiative, is worth $500,000 per year for 5 years. Last fall, the agency got a tongue-lashing from scientific societies and individual scientists because none of the nine winners in the first round was a woman (*Science*, 22 October 2004, p. 595). Only about 20% of the more than 1300 applicants were women, notes Judith Greenberg of the National Institute of General Medical Sciences, who is running this year's competition.

The new solicitation* says women and underrepresented groups "are especially encouraged" to apply by the 1 April deadline. NIH also hopes to diversify the pool of reviewers, 94% of whom were men. "I've been impressed by how quickly they've responded to the concerns," says Stanford University neuroscientist Ben Barres, a vocal critic of the first competition.

–JOCELYN KAISER

*grants.nih.gov/grants/guide/notice-files/NOT-OD-05-021.html

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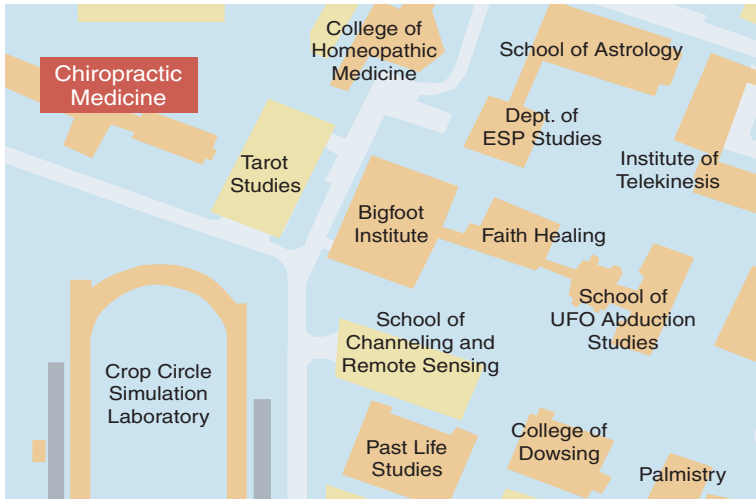
ACADEMIC AFFAIRS

Plan for Chiropractic School Riles Florida Faculty

Faculty members are questioning a plan to make Florida State University (FSU) in Tallahassee the first public U.S. university with a chiropractic medicine school. This week the faculty's graduate policy committee voted to examine the proposal amid concerns that implementing it would sully the university's reputation. But FSU administrators say such a graduate program, if ultimately adopted, would be a valuable addition to health care education and could benefit millions of Americans who suffer from back pain.



Realignment. This fictitious map of FSU's main campus, by chemist Albert Stiegman, has helped rally faculty opposition to a chiropractic school.

"There's a very good reason why no public university offers a degree in chiropractic medicine," says Raymond Bellamy, director of orthopedic surgery at FSU's Pensacola campus and leader of the opposition campaign. "It's because having a chiropractic program would seriously undermine the scientific tradition of any institution." Not so, says FSU provost Larry Abele, an invertebrate morphologist: "A graduate education and research program aimed at moving chiropractic medicine into a scientific and evidence-based realm is certainly worth exploring." The flap is reminiscent of a dispute at York University in Toronto, Canada, when faculty members blocked a plan to offer an undergraduate degree program that would have been affiliated with the Canadian Memorial Chiropractic College (*Science*, 19 February 1999, p. 1099).

Last March, at the urging of a state senator who's also a chiropractor, the Florida legislature authorized $9 million per year to establish such a school. FSU administrators conducted a feasibility study and drew up a proposal for a College of Complementary and Integrative Health that would offer a 5-year Doctor of Chiropractic degree. That proposal, which cited studies that it claimed showed "why more than 15 million Americans use chiropractic care," was to be presented this week to the university's board of trustees and 2 weeks later to the state Board of Governors.

Abele says chiropractic medicine is a legitimate field of study that deserves a place in the academic mainstream. He also says the university will not implement the proposal unless it has the support of the faculty: "The legislation simply authorizes funds for setting up the school. It does not require that we do so." Even so, FSU officials advertised in November for the position of dean of the proposed school.

Richard Nahin, a senior adviser at the National Center for Complementary and Alternative Medicine at the National Institutes of Health, says the popularity of chiropractic care among Americans makes it important to understand whether "chiropractic works, what conditions it may work for, and how it may work. Having a state chiropractic school could be of benefit to the field," he adds, "as that school would probably educate chiropractors using the same scientific, evidence-based approach used to train medical doctors."

None of those arguments is enough to convince neuroscientist Marc Freeman, one of 40 FSU professors—including Nobel Prize–winning chemist Harry Kroto and physicist J. Robert Schrieffer—who have signed a petition against the proposal. Apart from the lack of a scientific basis, he says, the chiropractic school is a threat to FSU's academic independence. "We cannot have the legislature forcing a program on a public university," he says.

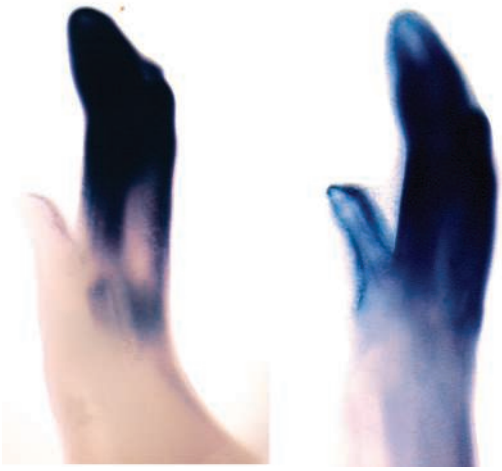
–YUDHIJIT BHATTACHARJEE

DEVELOPMENTAL BIOLOGY

Bird Wings Really Are Like Dinosaurs' Hands

Molecular studies have smoothed a wrinkle in the assumption that modern birds had dinosaur ancestors. After tracing the expression of two genes important in the development of digits in wings and other limbs, researchers have concluded that the three digits in bird wings correspond to the three digits in dinosaurs' forelimbs. For years, most embryologists had considered them different. "This may settle a long-standing controversy and will strengthen the therapod [dinosaur]–bird link," says Sankar Chatterjee, a paleontologist at the Museum of Texas Tech University in Lubbock.

Over the past decade, new fossils and phylogenetic analyses have convinced most paleontologists that birds are dinosaurs. A few researchers have refused to accept this evolutionary pathway, and one tenet of their argument has to do with how to count fingers.



Telltale tracers. The initial digits in developing wings arise where *Hoxd13* is expressed (right, dark stain) and *Hoxd12* isn't (left, dark stain).

Terrestrial vertebrates typically have five fingers, numbered 1 to 5. In both dinosaur fossils and birds, just three of these digits are fully developed, a trait that at first glance supports a dinosaur-bird connection.

But dinosaur forelimbs have the first three digits, with stubs for the last two. In contrast, going by some embryological evidence, birds appear to have retained the middle three fingers. In 1997, for example, ornithologist Alan Feduccia, a noted critic of the bird-dinosaur link at the University of North Carolina, Chapel Hill, and a colleague tracked digit ▶

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formation in turtles, alligators, ostriches, cormorants, and chickens. They concluded that the bird "fingers" were the middle three, whereas the reptiles' were the first three out of those five possibilities (*Science*, 24 October 1997, p. 666). That inference fueled arguments against a dinosaur-bird connection. In 1999, Yale University's Gunter Wagner and Jacques Gauthier, proposed a controversial compromise: that in avian ancestors, developmental signals transformed tissue in position to become digits 2, 3, and 4 into digits 1, 2, and 3.

Determined to resolve the issue, Alexander Vargas, an evolutionary-developmental biologist at the University of Chile in Santiago, and John Fallon, a developmental biologist at the University of Wisconsin, Madison, compared the embryological development of digits of mice and chickens. Working in Fallon's Wisconsin lab, they traced the activity of two genes crucial for digit development, *Hoxd13* and *Hoxd12*. Fallon and others had already shown that among other differences, the development of the first digit in mice relies on *Hoxd13* but not *Hoxd12*, whereas the other digits need both. The first digit also forms differently. "There are several molecular and developmental reasons to consider that digit 1 is distinct from other digits," says Vargas.

When the researchers looked at the chick embryo, they found that the wing's initial digit—until now considered to be digit 2, especially by opponents of the bird-dinosaur theory—used *Hoxd13* but not *Hoxd12*, indicating that it really is the first digit, developmentally speaking. Birds therefore have the same digits as dinosaurs, Vargas and Fallon conclude in the January issue of *The Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*. In birds, the first digit is simply masquerading as the second one. "I think it's the best evidence yet that digits gain their identities from [their genetic milieu] and not from position," says Richard Prum, an ornithologist at Yale University.

Friesten Galis, a functional morphologist at Leiden University in the Netherlands, is not convinced. Studies of digit development in other animals do not show as clear a difference in *Hoxd13* and *Hoxd12* expression as Vargas and Fallon presume, he points out. Galis cites new evidence he's recently obtained by studying birds with abnormal digit patterns that continues to support the idea that the digits in bird wings are equivalent to digits 2, 3, and 4 in other animals. And Feduccia is even more skeptical about the study and its conclusion. Hand development is just not that malleable, he insists.

The flap over bird wings continues.

–ELIZABETH PENNISI

CREDIT: NOAH BERGER/AP PHOTO

STEM CELLS

California's Bold $3 Billion Initiative Hits the Ground Running

Controversy over California's new stem cell initiative didn't end when the state's voters approved Proposition 71 in November by 59% to 41%. But now that the new California Institute for Regenerative Medicine (CIRM) is beginning to take shape, the debate has shifted from ethics and costs to how the enterprise will operate. Supporters are still brimming with confidence, however.

The new institute as yet has no staff, no home, and just a one-page Web site (www.cirm.ca.gov). But at a press conference last week, Robert Klein, CIRM's newly elected chair of the board, repeated assurances that he expects grants to start flowing by May. "I admit that I am an optimist," he added.

Committed father. Newly anointed stem cell czar Robert Klein with son Jordan.

At its first full meeting, held on 6 January at the University of Southern California in Los Angeles, the 29-member board, called the Independent Citizen's Oversight Committee (ICOC), set up subcommittees to find outsiders for "working groups" that will establish policies on research funding, ethics, and facilities construction. They also launched the hunt for a president for CIRM—ideally a seasoned research administrator who will be in charge of recruiting scientific advisers, directing staff, and participating in the formation of policies from lab construction to intellectual property agreements. Klein will head the search.

At the meeting, ICOC also elected as Klein's vice chair Edward Penhoet, a chemist who has straddled many sectors as a Berkeley dean, co-founder of Chiron Corp. in Emeryville, California, and most recently as president of the Gordon and Betty Moore Foundation in San Francisco. As a scientist and public health expert, Penhoet has a "complementary set of skills" to Klein's, says ICOC member Edward Holmes, dean of the University of California, San Diego, Medical School. Penhoet is heading the search for space for the institute's administrative headquarters. Also on the front burner is securing a start-up loan of $3 million from the state.

The critics have been busy as well. A primary concern, voiced by the Center for Genetics and Society in Oakland, among others, is that the initiative—which is immune from legislative tampering for the first 3 years—has been framed so that it may freely violate state and federal regulations on matters such as open meetings and conflicts of interest. Critics also worry that taxpayers won't get proper returns from patent and royalty fees, and some are troubled that Klein designed the entire initiative and slid into the top job without a hint of competition.

But supporters seem to have limitless confidence in 59-year-old Klein, who put more than $3 million of his own money into the Proposition 71 campaign and helped raise more than $20 million. A graduate of Stanford law school and president of Klein Financial Corp. in Fresno, California, which finances the construction of low-cost housing, Klein was drawn into the stem cell issue because his 14-year-old son Jordan has juvenile diabetes.

Committee members say they can negotiate the ethical minefield. "Whatever connections we might have anywhere" have to be a matter of public record, notes Holmes. Klein has pledged not to hold investments in biomedical or real estate enterprises "reasonably likely to benefit" from the stem cell program. He plans to step down after serving 3 years of his 6-year term. And he has resigned as head of the California Research and Cures Coalition (CRCC), which has been reconstituted as a nonprofit education and lobby group. CRCC hopes to build confidence with four community forums to be held around the state this month, at which citizens will discuss "practical and ethical issues" with scientists.

For now, at least, supporters seem to outweigh critics. "I think [the organizers of the CIRM] are drawing in the best this country has to offer," says Michael Manganiello of the Christopher Reeve Paralysis Foundation. Some scientists have expressed skepticism about the wisdom of funding research by means of popular vote and worry that the public has been oversold on the promises of the research. But it's hard to find a critic among stem cell researchers, who stand to benefit from the $3 billion and the new wave of attention that CIRM will bring to their field.

–CONSTANCE HOLDEN

After the discovery that several popular medicines may have harmed tens of thousands of people, experts are hunting for better ways to monitor drugs on the market

Gaps in the Safety Net

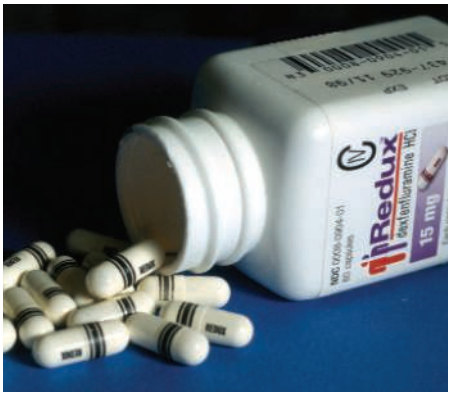
For those who trust government-approved drugs, 2004 was not a banner year. Merck, the maker of the anti-inflammatory medicine Vioxx, pulled the drug off the global market in September after a clinical trial linked it to heart attacks and strokes. In October, U.S. regulators concluded that a class of antidepressants can trigger suicidal thoughts in children and stepped up warnings of this danger. In December, studies of Celebrex, another arthritis medication, pointed to more cardiac risks. Just 5 days before Christmas, scientists running an Alzheimer's prevention study announced that Aleve, approved as a nonprescription painkiller in 1991, may also trigger heart problems.

These cases all involved drugs that had gone through extensive safety testing and had been on the market for years. And they raised disturbing questions: Should public authorities like the U.S. Food and Drug Administration (FDA) rethink what they consider acceptable risk? Should they move more aggressively to monitor approved drugs and restrict their use when problems surface among a fraction of patients?

The crises of 2004, some observers say, could trigger a shakeup in how drugs on the market are monitored. "I would like to believe that Vioxx could do for this decade what thalidomide did for the 1960s," says Jerry Avorn, a pharmacoepidemiologist at Harvard Medical School in Boston and author of the book *Powerful Medicine: The Benefits, Risks, and Costs of Prescription Drugs.* In the 1950s and 1960s, women in 46 countries who took thalidomide for morning sickness gave birth to more than 8000 children with severe abnormalities. Governments worldwide passed legislation requiring meticulous safety tests before a drug could be approved.

Judging by the numbers, the Vioxx case should elicit at least as strong a response. David Graham, an FDA drug safety officer, says it may have caused 100,000 heart attacks and strokes, a third of them fatal. Regulators from France to New Zealand had nervously discussed "signals" hinting at harm caused by the drug before 2004 but were unable to nail down their suspicions. It took a company-sponsored clinical trial to accomplish that (*Science*, 15 October 2004, p. 384).

Since the Vioxx debacle, officials running postmarketing surveillance systems are considering how they might do better. The uncomfortable truth, some say, is that all such systems have gaps. Several nations and the European Union (E.U.) boast aggressive surveillance systems, but many are new and have not been rigorously tested. "Everybody's in bad shape here," says Bert Leufkens, a pharmacoepidemiologist at the University of Utrecht in the Netherlands and an adviser to the Dutch and European Union drug agencies.



Same pill, different policies. FDA approved the diet drug dexfenfluramine, marketed as Redux, as European nations restricted access to it.

No public system is under greater pressure than FDA. Some members of Congress want to change it. Senator Charles Grassley (R–IA) plans to introduce legislation early this year to make FDA's existing Office of Drug Safety (ODS)—which is responsible for tracking the safety of drugs once they reach the market—independent of the drug approval mechanism in the Center for Drug Evaluation and Research (CDER), where ODS now resides. Academics and a few industry people say ODS needs a stronger legal mandate and more funds—but to make this happen, they must persuade a White House and Republican Congress that has traditionally recoiled from hands-on drug regulation.

Postmarketing surveillance systems, however, run on more than a legal mandate. Some of the strongest critics of the U.S. approach, like Avorn, say that FDA has all the police power it needs; it just needs to apply it creatively.

Risk tolerance

Forty years ago, European countries seemed relatively relaxed about drug approvals in contrast to FDA, which had earned a reputation for caution. Europe released thalidomide onto the market in the late 1950s, for example, and left it there for years. But an FDA reviewer spotted potential problems; she declined to let thalidomide through, and it was not approved.

Today, the roles are often reversed: FDA is frequently the first to approve drugs. The FDA staff is paid in part by "user fees" from regulated companies. Industry and patient groups lobby for speedy decisions, and FDA now turns some applications around in 6 months.

FDA has allowed greater risks in recent years than some other regulatory agencies, according to observers such as Lucien Abenhaim, a pharmacoepidemiologist at the University of Paris and McGill University in Montreal, Canada. He recalls getting little attention when he flew to Washington, D.C., in 1995 to warn FDA about life-threatening heart and lung ailments associated with the diet drug duo fenfluramine and dexfenfluramine (fen-phen). A recent study Abenhaim led had suggested that they increased cardiopulmonary risks up to 23-fold; European governments responded by limiting access to them. But FDA approved dexfenfluramine "without proper warning," says Abenhaim, only to see the drugs withdrawn in haste a year later after more than 100 people developed cardiopulmonary abnormalities.

Critics also fault FDA for its handling of the diabetes drug Rezulin. Two months after approving it in 1997, U.K. regulators pulled it off the British market because of concerns about liver failure. FDA read a different risk-benefit calculus in the data. "Most every country on Earth pulled the drug 2 full years before the FDA did," says Avorn.

Graham, a career FDA employee, claims that pressure to move faster has made CDER a "factory" for approving new drugs. Graham recently made headlines when he asserted in a Senate hearing that consumers "are virtually defenseless" against a repeat of the Vioxx affair. He said in a later interview that "my experience with FDA has been that

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they don't have the will" to go after drugs with safety issues. Graham says ODS, where he works, is often shunted aside because its views on a particular drug may threaten the judgment of FDA officials who allowed that drug on the market.

In an e-mail, FDA's press office declined to make senior officials available to answer questions for this article.

Shy gorilla?

Despite its woes, FDA remains a world leader in some areas—suggesting, perhaps, how tough it can be to police approved medications. "In many ways, the FDA is better able than we are at the moment to support independent research relating to pharmcovigilance," says Panos Tsintis, head of pharmacovigilance, safety, and efficacy at the 25-member European Medicines Agency (EMEA), the E.U.'s London-based drug approval and surveillance agency formed in 1995. Abenhaim praises FDA for its expertise but thinks these talents are poorly applied to postmarketing surveillance. He attributes this to government policy that gives FDA little authority to aggressively track and test marketed drugs.

Like agencies in many industrialized countries, FDA has two methods of conducting postmarketing surveillance. One is to commission specific studies. The other is to gather spontaneous reports of adverse effects in a database called MedWatch. Britain's drug regulatory agency claims to have the "world's largest computerized database of anonymized patient records," the General Practice Research Database (www.gprd.com). It's a fantastic research tool, says professor of medicine policy Joe Collier of St. George's Hospital Medical School in London—if you have a specific question and can pay. Full access to GPRD costs $600,000 a year.

No system is without flaws. One weakness of FDA's MedWatch, notes drug safety expert Alastair Wood, associate dean at Vanderbilt University in Nashville, Tennessee, is that it only skims the surface. He estimates that the 22,000 adverse events that are reported to the database each year represent only 3% to 10% of those experienced by patients. And the source could be biased: More than 90% of the reports come from companies, which are required to hand over reports given them by doctors, and fewer than 10% from doctors directly, FDA says.

Furthermore, FDA's MedWatch is isolated from patient care. In parts of Europe, "pharmacovigilance" offices are housed in hospitals, and physicians can wander down the hall to report adverse events. "It's not … an office somewhere in [FDA] with 8000 people collecting data," says Leufkens.

Then there's New Zealand's Medsafe, which employs 10 people on a budget of under $1 million to oversee more than 10,000 drugs on the market. Seventy percent of adverse-event reports to Medsafe come from general practitioners, 20% from hospitals, and 10% from companies. Those who submit reports can expect to hear from a Medsafe employee who's hunting for additional details. According to the World Health Organization, New Zealand's reporting rate on drug adverse effects is among the top three worldwide, says Stewart Jessamine, a Medsafe spokesperson.

New Zealand's challenge is very different from FDA's: The country has just 5000 prescribers and 3.5 million people. That makes it both easier to staff an interactive surveillance network and tougher to detect signals from dangerous drugs because fewer people are ingesting them, says Jessamine. Medsafe was watching Vioxx, for example, but officials could only conclude that "there's something happening, but we don't know what it is," he says.

This reflects the glaring limitation of even the best event-based reporting system: Doctors only report rare ailments that are easily linked to a drug. Vioxx and the heart attacks it induced are a different story altogether. "The doctor says … Mr. Blogg died from a heart attack, but he was 80, he did have angina and high blood pressure," says Jessamine.

No confidence. FDA's David Graham says the agency's system for protecting consumers from unsafe drugs is "broken."

Active surveillance

There are few ways to detect common but deadly hazards. One is through a clinical trial, like the one that brought down Vioxx. Another is by means of an epidemiology study that relies on massive databases, the kind maintained by HMOs such as Kaiser Permanente or government-funded health plans like Medicaid. Even though studies using these databases are cheap compared to clinical trials, running about half a million dollars, not many agencies fund them, says Brian Strom, a biostatistician and epidemiologist at the University of Pennsylvania in Philadelphia. Results from epidemiology studies sometimes carry less weight than those from clinical trials: Graham spent

Investing in Surveillance

	Total Staff	Postmarketing Staff	2004 Budget	Postmarketing budget
FDA-CDER (U.S.)	1800	94	$486 million	$24 million
EMEA (European Union)	300	55	$130 million	not available
Netherlands	130	25	$23 million	$3.5 million
New Zealand	50	10	$4.5 million	$900,000
United Kingdom	823	63	$125 million	$6 million

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3 years working with Kaiser in California on an epidemiology study of Vioxx and came to much the same conclusions as Merck eventually did, but his findings didn't prompt action against the drug.

FDA generally relies on companies to run postmarketing trials, called phase IV studies, often requesting them as a condition for a drug's approval. But follow-through is poor, a failing some blame on insufficient funds and others on a reluctance to confront drug companies. An FDA analysis released in 2003 found that more than 50% of phase IV studies don't even get started. FDA officials have said they need congressional authority to force companies to complete such studies.

Graham and Avorn think FDA has more muscle than its officials admit. If the FDA chief announced publicly that "there's a signal from Vioxx, the company's not responding," says Avorn, "the mere threat would have been enough" to force a clinical trial. The remedy, he and others say, is to give the drug safety office more clout.

Senator Grassley is proposing that the office remain within FDA but be distinct from CDER—a structure similar to that of the U.K.'s Medicines and Healthcare Products Regulatory Agency, in which safety regulators don't mingle with those who approve drugs.

Acting CDER chief Stephen Galson and other senior FDA officials declined to comment on FDA's postmarketing surveillance. But Jane Henney, FDA commissioner from 1998 until 2001 and now senior vice president and provost for health affairs at the University of Cincinnati, disagrees with Graham that FDA puts safety on the back burner, although she acknowledges that there will always be disagreement about how to handle drug risks. "As long as I was at the agency, the office of safety had a strong voice at the table," she says. Henney attributes FDA hesitancy to a simple problem: lack of resources. "We made a number of requests" to both Congress and the White House for increases in postmarketing surveillance funding, she says. Proposed changes included expanding FDA's access to large HMO databases to get a better grasp on adverse drug reactions and investing in research to more nimbly detect hints of drug problems. "Unfortunately, we just never got the money," says Henney.

Today, FDA devotes 5% of CDER funds, about $24 million, to the center's drug safety office, a fraction on par with the United Kingdom but proportionally lower than some other countries (see table, p. 197). Experts in both the United States and Europe believe that their countries should earmark far more money for postmarketing surveillance.

But money works best when melded with creativity. Even if FDA's drug safety office is refurbished, pressing postmarketing studies into action could mean flexing muscles drug regulators aren't accustomed to exercising.

Amid some controversy, France launched a new surveillance program several years ago that was spurred by the approval of Vioxx and Celebrex. EMEA had approved the drugs across Europe, but Abenhaim, then France's director general of health, wasn't convinced they worked as well as promised. He requested that a 2-year study of 40,000 people on Vioxx, Celebrex, or traditional nonsteroidal anti-inflammatory drugs begin before allowing France's national health care system to reimburse for the drugs. Abenhaim's position provoked an outcry, and he was asked to explain his position to the country's national ethics committee. In the end, the study was done. Since then, 50 more drug studies have been ordered. But, says Abenhaim, "there is still a lot of reluctance." Nor is the system efficient: The Vioxx study, for example, has not yet been released.

The Netherlands is eyeing a similar surveillance framework, says Leufkens. Meanwhile, EMEA, eager to harmonize drug approvals in Europe, will launch its own system in November 2005 to compel studies, using punishments such as financial penalties, says Tsintis.

The greatest worry of those pressing hardest for change, particularly in the United States, is that even thousands of possible deaths due to Vioxx won't prompt an overhaul of postmarketing drug surveillance. "My fear," says Avorn, "is that we will not be able to take advantage of this moment." —JENNIFER COUZIN

Radiation Hazards

Kyrgyzstan's Race to Stabilize Buried Ponds of Uranium Waste

With help from the West, local experts are devising ways to head off a potential landslide of Soviet-era mine tailings

MAILUU-SUU, KYRGYZSTAN—Alexander Meleshko scrambles up a terraced hillside, skirting tons of gravel laid to buttress the slope. All seems quiet on a cool day in late autumn, but Meleshko, a geologist with Kyrgyzstan's Ministry of Ecology and Emergency Situations (MEES), knows that this tranquil setting in the southwestern corner of the country is a disaster waiting to happen. Looming above is a 250-meter-high sandstone ridge rippled with shades of brown, yellow, and ochre. In front, entombed in an artificial hill, are 115,000 cubic meters of slurry chock-full of radioactive metals—enough to fill a football stadium. The noxious cocktail includes isotopes of thorium, copper, arsenic, selenium, lead, nickel, zinc, radium, and uranium. Meleshko, decked out in Army fatigues, stamps a foot on the soil. "There's more than 10,000 microroentgens per hour of radioactivity under here," he says—roughly 1000 times the local background rate.

High anxiety. Alexander Meleshko has charted a heightened landslide risk for Mailuu-Suu.

CREDIT: R. STONE/*SCIENCE*

All that protects Meleshko and the surrounding region from the tailings in this impoundment (called T-3), a leftover of Soviet-era uranium mining, is a meter-thick layer of clay. Experts have identified T-3 as a far-reaching threat: In the scariest scenario, the ridge could dissolve in a landslide, sweeping the tailings into the nearby Mailuu-Suu River. That's a chilling possibility. The Mailuu-Suu is a tributary of the Syr Darya River, the main source of irrigation water for the 6 million residents of the densely populated Fergana Valley. "It's a huge potential danger," says Vyacheslav Aparin, a senior scientist with the Complex Geological-Ecological Expedition in Tashkent, Uzbekistan. The valley, which extends southwest into neighboring Uzbekistan and Tajikistan, is a melting pot of peoples and beliefs, including enclaves of Islamic fundamentalists. A radioactive accident here could be traumatic to a region already simmering with tension.

The risk of a catastrophe is rising. Heavy spring rains in recent years have made landslides a more frequent occurrence in mountainous Kyrgyzstan, and in this seismically active

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Big trouble in little Kyrgyzstan. Major sites of Soviet-era uranium tailings are an enduring legacy of the Cold War.

region, a tremor capable of unleashing a devastating landslide could strike at any time. "There's not much we can do if there's a strong earthquake," says Isakbek Torgoev, director of the Geopribor engineering center in Kyrgyzstan's capital, Bishkek. Tajikistan and Uzbekistan are also grappling with the legacy of Soviet uranium mining. Anecdotal reports suggest that some sites in Tajikistan are in an even more precarious state than those in Kyrgyzstan.

But Mailuu-Suu, poised like a match near Fergana's tinderbox, is deemed the top priority. After years of handwringing, Kyrgyz authorities are on the verge of doing something. In September, Kyrgyzstan received the first installment of a $6.9 million World Bank loan to deal with the most hazardous uranium sites, starting with T-3.

Work could begin as early as next summer—which would be none too soon. Authorities will be pacing anxiously when meltwater and rain renew their assault on the fragile land in the spring. "In our narrow valleys, gravity wins sooner or later," says MEES's Nurlan Kenenbaev.

Bad to the bone

When the Soviet Union pushed its atomic bomb program to full throttle after World War II, Mailuu-Suu, nestled in the foothills of the Tian Shan mountains, was wiped off maps and became known simply as P.O. Box 200. Specialists arrived here in droves.

Officials in faraway Moscow pampered their uranium jocks with high salaries and ample food trucked in even during lean times. "The standard of living was much higher than it is today," says longtime resident Ashir Abdulaev, an assistant mayor of Mailuu-Suu and local MEES representative. But many in Mailuu-Suu and other uranium towns in Central Asia had no idea why they were so well off. Operated by the Ministry of Medium Machine Building, which ran the bomb program, the uranium facilities "were top secret," says Alexander Kist, a radiochemist at the Institute of Nuclear Physics in Tashkent. According to Torgoev and others, the first Soviet bomb was made from uranium milled at Mailuu-Suu.

In those days, says Aparin, "there was no such science like ecology, so the idea was to just get the uranium out of the ground as fast as possible." Nazi POWs and prisoners from Tatarstan, Ukraine, and elsewhere toiled in shafts laden with radon, a radioactive gas that wafts from the ore. "They didn't know what they were mining," says Torgoev. Even the miners' housing was built from uranium-rich stone. (According to Kist, the skeletal remains of workers are radioactive.) Lavrenti Beria, one of Stalin's most feared henchmen and chief of the bomb project, would come to Mailuu-Suu to check on the mines. Today his former quarters, garishly decorated with yellow and blue plastic wall tiles, is part of a hotel.

Most people connected with the mines have left or died, but reminders of Mailuu-Suu's past linger. Tidy, two-story stone houses, built by German prisoners for the town's elite, line a street leading to a pair of former uranium mills. One mill was converted to a factory, Isolite, which makes insulation materials and glass wire. The other mill is a heap of rubble. The Soviets abandoned it in the 1960s after radioactive contamination of the machinery had grown intolerable even by the lax standards of the day, Meleshko says. Rather than dismantle the site, the Soviets blew it up. These days, locals have been seen scavenging tainted metal from it.

If anything, the shadows in Mailuu-Suu are deepening. Its population has dwindled from 36,000 to 23,000, in part due to an exodus after the uranium industry shut down. Local health officials assert that radioactive contamination is killing off many who stayed behind. "The cancer rate here is twice that of the rest of the republic," claims Nemat Mambetov, chief of Mailuu-Suu's Sanitary and Epidemiological Station. Lung cancer is the biggest killer, he says, followed by stomach and digestive tract cancers—although he acknowledges that limited financing has resulted in poor record-keeping. Western experts are circumspect. "We've had trouble getting reliable epidemiological data," says Peter Waggitt, an expert on uranium tailings with the International Atomic Energy Agency in Vienna. "You can't automatically just blame every cancer on the uranium."

In 1958, flooding after a landslide ate into one of the impoundments at Mailuu-Suu (T-7), sweeping an estimated 300,000 cubic meters of tailings into the river, says Yuriy Aleshin, a geophysicist with Geopribor. The tailings, he says, are thought to have spread tens of kilometers downstream. The consequences of the accident may never be known: Soviet authorities hushed it up, and records of any follow-up studies have long since disappeared.

From qualitative analogies with Cold War–era tailings sites in the United States, Richard Knapp, a geoscientist with the Proliferation and Terrorism Prevention Program at Lawrence Livermore National Laboratory in California, has come up with a preliminary estimate of the potential risk posed by T-3: If it were to disgorge its contents today, the contamination would cause about 600 cancer deaths in the vicinity of Mailuu-Suu over 100 years, he estimates. In contrast, a 25-year cleanup at two dozen U.S. tailings sites has prevented about 1300 deaths combined, Knapp says.

In harm's way. The Isolite factory, a former uranium mill, is in line for a direct hit from a landslide.

CREDITS (TOP TO BOTTOM): ADAPTED FROM KYRGYZSTAN MINISTRY OF ECOLOGY AND EMERGENCY SITUATIONS; R. STONE/*SCIENCE*

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A uranium rust belt

Kyrgyzstan is not alone in its woes. Next door in Uzbekistan, the major headache is Charkesar, a fenced-off, decommissioned uranium mine that Aparin and others say may have sickened thousands of local residents. Uranium mining is still a big business there, unlike in Kyrgyzstan. These days, however, companies rely on a sulfuric acid process rather than miners to extract ore.

Tajikistan too was a major uranium producer in Soviet times. Processing took place at three sites: Adrasman, Chkalovsk, and Taboshar. According to a 2004 report from the state mining enterprise Vostokredmet, twice in recent years mudflows have destroyed impoundments at Taboshar. One Western expert who has visited the site describes having seen "mountains of tailings," one 200 meters high, in the open air. Tajikistan will host a workshop in May, sponsored in part by the U.S. Department of Energy, to highlight the region's problems and attract international donors.

Nor is Mailuu-Suu the only worry for Kyrgyzstan. Another 12 hot spots are scattered across the country. After the Soviet breakup in 1991, says MEES Director Anarkul Aitaliev, "no maintenance was done on the tailings." The U.S. State Department is funding a $500,000 effort, led by Lawrence Livermore with support from Russia, to deal with the Kadzhi-Say impoundment on the south shore of Lake Issyk-Kul. Kyrgyzstan has staked its development on tourism, and the lake is its biggest asset. "Anything that jeopardizes Issyk-Kul is a concern," says Knapp.

But the consensus of international agencies is that Mailuu-Suu poses the biggest risk. "Mailuu-Suu is critical because at the end of the road is another country," says Waggitt.

Exacerbating the situation is that the environment is literally falling to pieces. Meleshko has charted a steady rise in the incidence of landslides in Kyrgyzstan, from about 100 major slides per year in the 1970s to more than 200 last year. Last year, 45 people in Kyrgyzstan died as a result of landslides, including 33 in a single disaster last April not far from Mailuu-Suu. The higher frequency of landslides has followed, almost in lockstep, seasonal increases in precipitation. "The more rain and snow, the more chance of landslides," Meleshko says.

In May 2002, a slide just a kilometer upstream from T-3 engulfed several Isolite buildings. Today, an estimated 5 million cubic meters of soil at the site are at risk of sliding down. Although it wouldn't plow into T-3 directly, such a landslide could lead to a replay of the 1958 incident at T-7, this time disemboweling T-3.

No-go zone? Grazing animals—and people—routinely ignore this sign warning of radioactivity near the T-3 uranium tailings impoundment near Mailuu-Suu.

Move it or leave it?

A fluke of Cold War political geography makes Mailuu-Suu—and T-3 in particular—more hazardous than other sites. From 1946 to 1967, more than 10,000 metric tons of uranium oxide were processed in Mailuu-Suu. Many more tons were shipped here for processing from Saxony, in eastern Germany, and elsewhere in the East Bloc. After some of the uranium was extracted, the leftover slurry was piped into the clay-lined impoundments. Tailings from the imported ore are hotter than those from local deposits, Torgoev says, accounting for a substantial fraction of the radioactivity sequestered in T-3.

Last year, thanks to a grant from the European Union, gravel was laid to shore up the base of the 20-meter-deep T-3. Now Kyrgyzstan is about to embark on a broader $16.7 million effort to clean up Mailuu-Suu. An initial $12 million from the World Bank, Japan, the Global Environment Facility, and the Kyrgyz government "will allow us to deal with the most dangerous parts of the problem," says Meleshko. The first step is to remove soil from the ridge above T-3 that's deemed especially prone to sliding down. With funds in hand, Kyrgyz authorities are now selecting contractors; work could begin as early as next summer.

T-3's ultimate fate is unclear. "It's very difficult to come up with a solution; it's a huge volume," says Meleshko. Complicating matters, the drainage system that prevented rain and groundwater from saturating the 50-year-old impoundment no longer works, says Knapp. Water percolating into T-3 explains why the tailings, which have the consistency of toothpaste or newly mixed cement, are unusually mushy—and unstable.

One option that Kyrgyz authorities are considering is to pump out the tailings from T-3 and store them at a more stable location nearby. Such a procedure has been carried out successfully in the United States. "About half of [the U.S. impoundments] were just picked up and moved somewhere else," says Knapp. He advocates this solution for T-3, as it would be almost impossible to eliminate a landslide risk. Some experts in Kyrgyzstan, including Torgoev, also favor this strategy. But there are risks: Such an operation could expose workers to increased radiation levels, and if an accident were to occur, says Aparin, "you could contaminate the whole valley." Also a big issue, says Waggitt, is where precisely to put the tailings. "If you look around the valley, there's an awful lot of instability in the landscape," he says.

The other option is to leave the tailings in place and sculpt the ridges to avert a serious landslide threat. Although a massive job, it might be considerably cheaper than hauling out the tailings, says Meleshko. Experts in Uzbekistan are pressing for a third option: installing a pipe to divert any floodwaters generated by a landslide upriver around the T-3 impoundment. "I see this as giving a 100% guarantee of success," says Vladimir Kupchenko, director of Uzbekistan's Complex Geological-Ecological Expedition.

It may take up to 2 years to make a decision and bring in new equipment and expertise, says Kenenbaev of MEES: "Everything we have is from the Soviet period."

In the meantime researchers must play a waiting game. Making a brief stop on the long road back to Bishkek, Meleshko admires a landscape that could have been painted by El Greco. Dark-gray clouds cling to the mountains, their snowcapped peaks and glacial fields glowing eerily white in the twilight. The treeless land stretches like crumpled brown velvet as far as the eye can see. But Meleshko can't tear his thoughts from Mailuu-Suu. "We've waited 40 years to do something about it," he says. "I hope nature will let us wait a few more months."

—RICHARD STONE

CREDIT: R. STONE/*SCIENCE*

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Failure to Gauge the Quake Crippled the Warning Effort

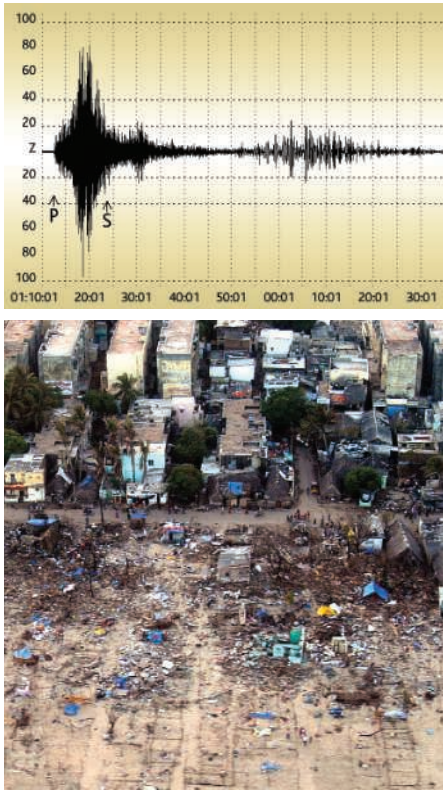
Seismologists knew within minutes that the earthquake off Sumatra must have just unleashed a tsunami, but they had no idea how huge the quake—and therefore the tsunami—really was

When 1000 kilometers of subsea fault ruptured that Sunday morning west of Sumatra, seismologists knew a tsunami was on the loose, but they failed to grasp the true magnitude of the quake and therefore the hugeness of the tsunami it had spawned. Measuring earthquakes is no easy task, and only a single, unstaffed lab on the other side of the world had the proper tool.

"Everybody underestimated [the earthquake] in the beginning," says Charles McCreery, director of the Pacific Tsunami Warning Center (PTWC) in Ewa Beach, Hawaii. That was because no seismologist was using the one, long-available technique that could nail down the magnitude of a truly great quake. Seismologists have long known that the commonly available methods underestimate any quake larger than about magnitude 8.5. The Sumatra-Andaman Islands quake turned out to be 9.0. That's 30 times stronger than initial estimates and was guaranteed to produce a deadly, far-ranging tsunami. A computer at Harvard University, using a mathematical technique called centroid moment tensors (CMT), automatically calculated a magnitude of 8.9 within 2 hours of the quake, but the results became available only when seismologists later checked its readout.

At PTWC, staffers calculating magnitudes from the seismic data circulating worldwide at first thought December's quake looked like a fairly run-of-the-mill magnitude 8.0. When the first informational PTWC bulletin went out 15 minutes after the quake, "there could have been a local [Sumatran] tsunami by then," says McCreery, but at 8.0, nothing damaging would ever make the 2-hour trip across the 1600 kilometers of the Bay of Bengal to India or Sri Lanka. So that first bulletin, sent to participating Pacific Rim countries that PTWC is mandated to alert, reported the 8.0 magnitude and the absence of any threat around the Pacific.

As more seismic data arrived, the quake's perceived size grew. The magnitude 8.0 estimate had come from a technique dubbed M_{wp}, which was designed for speed and used some of the first seismic waves arriving at seismometers. But speed had a drawback. With M_{wp}, the rupture is assumed to be a one-dimensional point. That works pretty well up to magnitude 7.5 or 8. However, faults rupture along planes, not at points, and a bigger quake can rip hundreds of kilometers along the fault. The P waves used in M_{wp} zip through the earth much more directly than seismic surface waves do, but surface waves paint a clearer picture of the full, two-dimensional extent of a great earthquake's rupture. After gathering a full hour of data including late-arriving surface waves, McCreery and his colleagues were confident they had a magnitude 8.5.



The wiggles knew. Only one technique for estimating the quake's magnitude got it right because it extracted more information from seismic waves.

So an hour after the quake—with the tsunami halfway across the Bay of Bengal—PWTC issued a second bulletin reporting the higher magnitude. Within minutes, the U.S. Geological Survey's National Earthquake Information Center (NEIC) in Denver, Colorado—the world's de facto seismic clearinghouse—sent out its own, independently calculated surface wave magnitude of 8.5 to its worldwide alert list. Any seismologist aware of the quake would now know it was underwater and sizable.

What that meant for the tsunami threat was unclear, even to McCreery and his colleagues. "Around 8.5 is when we start to feel there's some kind of reasonable threat" at greater distances from the quake, says McCreery, "but it's not consistent." Lacking a system of sea-floor sensors to detect and gauge tsunamis in the Bay of Bengal, "we felt pretty frustrated," he says. But "none of us was thinking it would be a 9," he adds, so PTWC's second bulletin merely noted "the possibility of a tsunami near the epicenter." Meanwhile, according to news reports, low-level scientists across Asia were passing word to superiors of a large, threatening underwater quake in the region, but their similarly vague warnings went unheeded.

Chances are that alarms would have traveled faster and farther if seismologists knew what a computer in Cambridge, Massachusetts, was learning. By the time the first waves hit India, it had automatically calculated a magnitude of a little over 8.9, according to Harvard seismologist Göran Ekström. That was 30 times more powerful than an 8.0 and easily large enough to produce waves that could damage India and Sri Lanka. The Harvard technique used not just the size of seismic waves but also their varying shapes, as recorded at varying distances and directions from the rupture. That extra information enabled the computer to gauge the true size of the fault rupture and thus the true magnitude of the quake, known as a CMT magnitude.

Ekström, then on vacation and away from his lab, logged in to the computer remotely after happening on an NEIC alert while checking his e-mail. Four-and-a-half hours after the quake, he and Harvard colleague Meredith Nettles e-mailed a recalculated magnitude to NEIC and PTWC. That was after India and Sri Lanka were hit but before the tsunami reached East Africa, where it killed more than 100 people.

If the Sumatran quake—which might recur once a millennium—had struck a year later, Ekström says, the world could have marked it as a killer more than an hour before it struck India. By then, under a USGS grant issued before the quake, NEIC will be receiving Harvard's automatic CMT analysis in real time 24/7. And a little fine-tuning can accelerate such real-time magnitude estimates to within three-quarters or even half an hour after a quake, says Ekström.

In the end, scientists did not have the fastest, most accurate warning tool at hand because no one had fully grasped the need. "We've known there was a problem" off Sumatra, says Bilham, but "I'm surprised out of my wits about the magnitude of it." It's clear now, he says, that "seismologists have to grapple with absolutely worst case scenarios."

–RICHARD A. KERR

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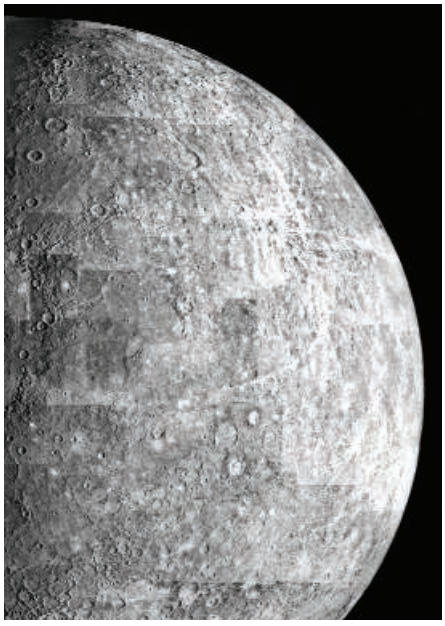
A Lively Core Turns Mercury Into An Enormous Electromagnet

San Francisco, California—More than 11,500 earth scientists from around the world gathered 13 to 17 December at the fall American Geophysical Union meeting to discuss everything from Mercury's core to the rings of Saturn.

By all rights, the Mariner 10 spacecraft should have found a geophysically dead planet when it flew by Mercury in the mid-1970s. But to everyone's surprise, Mariner detected a weak magnetic field emanating from the sun's closest companion. A still-molten iron core churns out Earth's field,



It's alive. Despite its lunarlike exterior, Mercury harbors a churning molten core.

but Mercury's field seemed too weak to be generated that way. And besides, planetary scientists thought Mercury's big iron core must have frozen solid eons ago. Alternatively, if an early field-generating core had locked its field into Mercury's crust before freezing up, the field would be much stronger than Mariner's discovery. No spacecraft has revisited Mercury, but at the meeting, two groups of researchers built a strong case that Mercury generates its magnetic field in a lingering remnant of a molten core, much the way Earth's geodynamo operates.

The trick to diagnosing Mercury's interior without leaving Earth was measuring the planet's rotation rate to 1 part in 100,000. A combination of asymmetries links Mercury's interior to its rotation, as planetary scientist Jean-Luc Margot of Cornell University explained in his presentation. Mercury itself is slightly egg-shaped rather than spherical, so the sun's gravitational pull tends to align a bulge of the planet sunward. But Mercury's orbit is elliptical, not circular, so the planet's orbital motion tends to drag it out of its sun-induced alignment. The sun then tugs Mercury back toward alignment, ever so slightly slowing its rotation rate. Further along in the planet's orbit, the sun speeds up the rotation rate.

The amplitude of this rotational slowing and speeding up, or libration, depends on how much of the planet the sun must tug on. If even just the outer core is molten, that would disconnect the interior from the rocky outer shell, greatly reducing the mass that must be realigned and increasing the amplitude of Mercury's libration to at least double that of an entirely solid body.

Margot and his colleagues used a previously proposed ground-based radar technique to precisely measure variations in Mercury's rotation during the past 2 years. They repeatedly beamed a radar pulse at Mercury from the 70-meter antenna at Goldstone, California, and picked up the reflected signal at both Goldstone and the 100-meter antenna at Greenbank, West Virginia, 3200 kilometers to the east. Matching up the distinctively "speckled" pattern in the signal received at each station, they gauged the time lag of reception between stations and thus calculated the rotation rate precisely. It varied with Mercury's 88-day libration three times as much as it would if the planet were solid throughout.

Given such a definitive result, "it looks as if [a molten core] is the only explanation," says planetary geophysicist David Smith of NASA's Goddard Space Flight Center in Greenbelt, Maryland. That still would leave the difficulty of why Mercury's magnetic field has only 1/100 the strength of Earth's geodynamo-generated field.

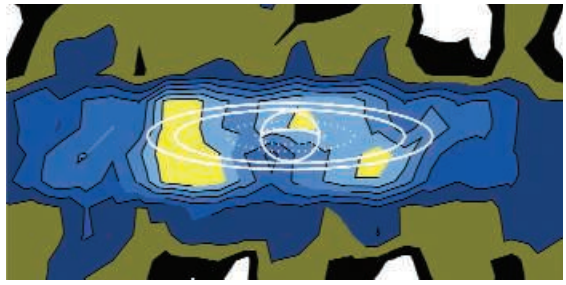
In a poster presentation at the meeting, planetary geophysicist Sabine Stanley of the Massachusetts Institute of Technology and her colleagues showed how the Mariner measurements could be misleading. They ran a computer model developed to simulate the geodynamo churning in the molten outer core of Earth, between a rocky mantle above and a solid-iron inner core within. On the assumption that Mercury's molten outer core had shrunk to a thin shell by now, they ran the model with progressively thinner outer cores. The model's dynamo continued to generate a relatively strong field within the core, but the field that it could project outside the core weakened to the point that a passing spacecraft would detect a very weak field even while a strong field dominated the core.

The Messenger spacecraft, launched last August, should be able to test the state of Mercury's core and the nature of its magnetic field after entering orbit in 2011.

What's Going On in Saturn's E Ring?

Saturn's faint, broad E ring encircles the planet beyond the main rings with no visible means of support; no one ever has figured out what it's doing there. And no one can figure out what it was up to late last winter, either, when it apparently spewed out a cloud of water equal to its own mass. Whatever created the E ring in the first place—collisions of stealth moonlets or eruptions of icy volcanoes on the moon Enceladus, perhaps—may be responsible.

The E ring outburst came just as the Cassini spacecraft approached Saturn, carrying its Ultraviolet Imaging Spectrograph (UVIS), an instrument well suited to map out the glow of oxygen atoms near Saturn. At the meeting, UVIS principal investigator Larry W. Esposito of the University of Colorado, Boulder, and Donald Shemansky of the University of Southern California in Los Angeles described how 500,000 tons of oxygen atoms appeared during 2 months as an ultraviolet glow in the UVIS images. The oxygen formed a doughnut-shaped ring engulfing the E ring, then faded just as rapidly, leaving Saturn's magnetosphere depleted of ions.



Ring cloud. A UV glow of oxygen (yellow and light blue) engulfs the orbits (white ovals) of Saturn's Enceladus and Tethys.

CREDITS (TOP TO BOTTOM): NASA/JPL; L. W. ESPOSITO *ET AL.*

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That sequence of events suggests to Esposito and Shemansky that half a million tons of water ice crystals were suddenly added to the E ring, which already contained an equal mass of 1-micrometer ice particles. Colliding energetic ions would have knocked oxygen atoms free of the newly released ice. The resulting neutral oxygen atoms could then pick up charge from magnetospheric ions and eventually be ejected from the saturnian system, leaving the E ring much as it was.

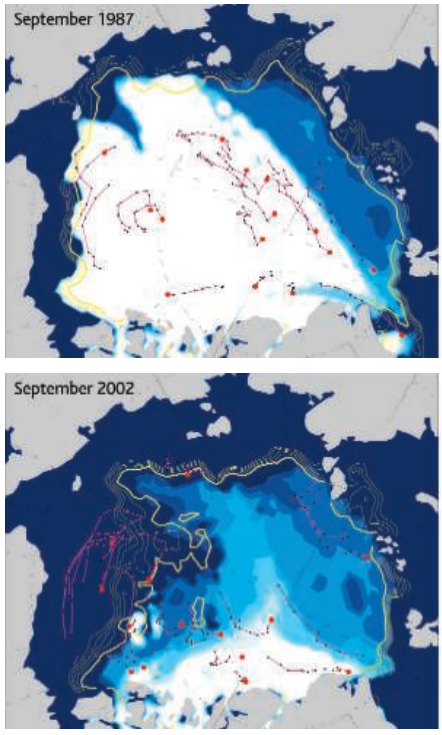
What could have injected that much ice into the E ring so suddenly? Esposito favors a catastrophic collision of two unseen icy bodies orbiting in the E ring. Such embedded moonlets sustain the faint ring of Jupiter, but they do it through continual erosion by impacting micrometeoroids, not by collisions among themselves. It would take an improbable coincidence or a great many embedded moonlets to explain a major collision just as Cassini approached. Ring specialist Joseph Burns of Cornell University doubts that there are enough E ring moonlets. A Cassini camera search for such bodies larger than 1 to 2 kilometers in diameter is 95% complete, he says, but none has been found.

Alternatively, the water might have been blasted off the moon Enceladus in a volcanic eruption. But the two Voyager spacecraft found no signs of ongoing eruption there in the early 1980s, although they did find plains that might have been smoothed by geologically recent watery volcanism. "You've got several bad alternatives," says Burns. Puzzled ring scientists hope that three Cassini close flybys of Enceladus this year, the first on 17 February, will improve their choices.

Scary Arctic Ice Loss? Blame the Wind

The past three Septembers have seen the Arctic ice pack shrink dramatically to a record low amid signs that greenhouse warming could be melting the ice, threatening to clear the Arctic Ocean within decades. Researchers are still worried, but a study presented at the meeting offers some reassurance. A natural, temporary shift in the wind may have been largely to blame for the recent shrinkage.

Winds of the high northern latitudes are the domain of the Arctic Oscillation (AO), an erratic atmospheric pressure seesaw (*Science*, 9 April 1999, p. 241). Over weeks, years, or even decades, pressure can fall over the pole while rising around a circle near the latitude of Alaska. The resulting steeper pressure drop across high latitudes increases the generally westerly winds blowing there. When the pressure seesaws the other way, the winds drop to weaker than average.



Ice lost. A wind-driven model loses much of its older, thicker Arctic ice (white) in 5 years.

Wondering how the AO had been influencing Arctic ice, meteorologists Ignatius Rigor and J. Michael Wallace of the University of Washington, Seattle, created a model that keeps track of ice as it forms and blows around the Arctic Ocean, thickening with time. In the 1980s, the AO was in its so-called low-index phase, with higher than average pressure over the pole and therefore weaker westerly winds. In the model, those winds tended to drive the ice around in circles off the Alaskan and Siberian coasts, giving it a chance to thicken for an average of 10 years or more. But in the 1990s, the AO swung into its strong-wind phase. In the model, the new circulation tended to blow old, thick ice out of the Arctic Ocean through the Fram Strait and into the North Atlantic. The remaining ice was thinner than under the opposite AO phase and thus easier to melt away. In fact, ice did surge through Fram Strait in the early 1990s, and the ice has thinned, culminating in the record low ice extents of recent years.

At least some of the recent ice loss is indeed "a hangover effect" of the early '90s swing in the AO, says meteorologist Mark Serreze of the University of Colorado, Boulder. The AO index fell back toward more normal levels in the late '90s, he notes, but the ice hasn't recovered yet. Because Arctic warming has been lengthening the period in the summer during which ice can melt, he says, Arctic ice may well continue to shrink, although probably not as rapidly as it did recently.

In the long term, Serreze adds, climate models predict that greenhouse warming should lead to increased melting over coming decades. Some models even have the intensifying greenhouse pushing the AO into a permanent positive phase, he says, which would favor still-greater ice losses.

–RICHARD A. KERR

CREDITS: I. RIGOR/UNIVERSITY OF WASHINGTON

Snapshots From the Meeting

No vestige of a beginning. Seismologists got their most detailed look at an earthquake last fall when 30 kilometers of the San Andreas fault ruptured through the town of Parkfield, California, and its dense array of instruments, but they still missed something. "This is the best data we've got," said geophysicist Malcolm Johnston of the U.S. Geological Survey in Menlo Park, California, but there is still no sign of the slow, hesitant onset of the fault rupture that some seismologists have been looking for (*Science*, 6 January 1995, p. 28). If earthquakes were to begin as slow slippage on a small patch of fault, well-placed instruments might detect it days or even weeks before the slippage took off and produced a quake. But the Parkfield data limit any such nucleation patch to a few tens of meters or less in size, says Johnston. So, even if nucleation occurs, detecting it looks improbable.

A nudge toward magnetic flip-flop. Two paleomagnetists found themselves presenting adjacent posters that argued for a previously unrecognized precursor to the most recent reversal of Earth's magnetic field. Researchers had thought that the field generated by the churning molten iron of the outer core had simply weakened and reorganized itself for a few thousand years as it got ready to flip about 775,000 years ago. Not so fast, say Laurie Brown of the University of Massachusetts, Amherst, and Bradley Singer of the University of Wisconsin, Madison. Brown, working on the paleomagnetic record frozen into lavas of central Chile, and Singer, studying lavas in Tahiti, found that the field had actually weakened and moved toward a reversal 18,000 years earlier. The prolonged precursory move toward reversal may have given the liquid outer core time to overcome the stabilizing influence of the solid inner core.

–R.A.K.

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Random Samples

Edited by Constance Holden

A Clean Sweep

A sticky situation with geckos has been resolved. The nimble little reptile's toes are so adherent that it can suspend itself by a single digit, yet its feet never get fouled up with dust. Now, using microscopic silica-alumina spheres, a physicist and a biologist at Lewis and Clark College in Portland, Oregon, have figured out why.

They dusted geckos' feet with the spheres and found that as the reptiles walked, their feet shed the spheres and quickly returned to peak stickiness. The spheres stuck to the surface more readily than they did to the feet because the electrostatic attraction of the surface is greater than the collective attraction of the tiny hairs on the toe pads, explain the scientists, Wendy Hansen and Kellar Autumn. So the pads naturally cleaned themselves as the lizards ambled about.

"It opens up the question, 'Can we repeat this with manmade materials?'" says Daniel Fletcher, a bioengineer at the University of California, Berkeley. A self-cleaning adhesive would obviously be useful, he says. This sort of research, Fletcher adds, might also help people figure out how to thwart infectious diseases by foiling microbial adhesives, such as the one that allows the diarrhea-causing parasite *Giardia lamblia* to stick to the walls of the intestine.

The gecko's self-cleaning footpads stay tacky.

Monumental Makeover

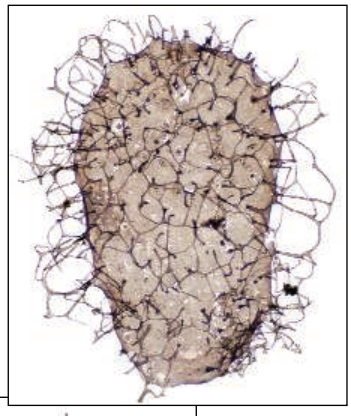
Brussels's most famous science monument is getting a facelift. The Atomium, a 102-meter-high model of iron atoms in a crystalline structure (magnified 165 billion times), has been part of the landscape since its construction in 1958 as part of the World Expo celebrations. But guests in recent years have noticed that the oversized tribute to the 1950s' faith in science and technology is looking increasingly tatty. The city of Brussels and the Belgian government are now contributing 70% of the $32 million needed to replace the aluminum and steel surface and update the interior where people look out from the windowed spheres and read yellowed posters about the wonders of atomic energy.

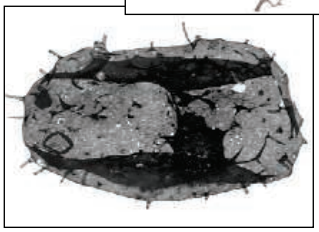
To help cover the rest, the 1000 old aluminum panels that covered the atoms are being sold to Atomium enthusiasts for €1000 ($1400) apiece. The monument, closed during the renovations, is expected to reopen early next year.

Primordial Fungus

Exquisite microfossils dissolved out of 850-million-year-old rocks could be from the most ancient fungi ever discovered.

Fungi, which are closer relatives to animals than to plants, have been conclusively identified as far back as 380 million years ago. The new fossils, which are no bigger than half a millimeter, were painstakingly sieved out of a slurry of dissolved shale from Victoria Island, Canada, by paleontologist Nicholas Butterfield of the University of Cambridge, U.K.





Most of the fossils have a rounded central body covered with multicellular filaments. The key feature, as Butterfield describes in the current issue of *Paleobiology*, is that these filaments join to form networks of loops—diagnostic of modern "higher" fungi. The fossils don't belong to any living group. But Butterfield says they resemble mysterious microfossils from China and Australia called *Tappania*, some of which are nearly 1.5 billion years old. "I can almost put my hand on my heart and say we've got a fungus at 1400 million years," Butterfield says.

Other experts say the evidence is strong, but not conclusive, that the Canadian fossils are fungi. Emmanuelle Javaux of the University of Liège, Belgium, a member of the team that first described the Australian fossils, thinks the two groups could be related. But she also notes that the older *Tappania* have different features and lack the joined loops, known as hyphal fusion. If fungal identities are confirmed by further studies, they would add substantially to the known diversity of early life and provide a new calibration point for the molecular clocks used to date major evolutionary events, says Butterfield.

CREDITS (TOP TO BOTTOM): W. HANSEN AND K. AUTUMN; NICHOLAS J. BUTTERFIELD; FAST FIX EURO LTD.

Published by AAAS

Edited by Yudhijit Bhattacharjee

JOBS

Is it contagious? The head of infectious diseases at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, is stepping down, the latest in a string of high-profile departures from the agency. Physician James Hughes, 59, who for 12 years has led CDC's National Center for Infectious Diseases (NCID), this spring will move to nearby Emory University to direct new international programs on safe water and infectious disease.

The directors of five other centers run by CDC have left in the past year. CDC spokesperson Thomas Skinner says they all were eligible for retirement. However, observers say other factors--such as new requirements for CDC staff in the U.S. Public Health Service commissioned corps and an ongoing reorganization by Director Julie Gerberding that groups CDC's 11 centers into "clusters" (*Science*, 30 April 2004, p. 662)—are contributing to the exodus. Some are worried, for example, that changes in budgets could harm CDC's infectious disease control efforts, notes a member of NCID's board of scientific counselors. But he adds that the reorganization "could be a positive thing if done correctly."

NCID is also losing its second in command, epidemiologist Stephen Ostroff, who is taking a job as a Department of Health and Human Services health attaché in Hawaii next month. Hughes was on medical leave and not available for comment.

Offering stability. Spain hopes to slow the exodus of young scientists by creating 900 new jobs at universities and nonprofit research centers over the next 3 years. The Science and Education Ministry says the positions will be permanent, unlike the temporary jobs offered under past initiatives aimed at reversing the country's brain drain.

State secretary of science policy Salvador Barberá says the ministry will provide up to $14 million a year in grants to regional governments to fund the plan. Scientists with 4 years of domestic or overseas postdoctoral experience will be eligible for the positions, which will emphasize research over teaching.

Biologist Arcadi Navarro, who joined Barcelona's Pompeu Fabra University in 2002 under a program with similar goals but no guarantee of permanent employment, welcomes the announcement but has a "lot of doubts" about whether the positions will truly be secure in the long term. He also thinks that 900 jobs may not be enough to make a difference.

HONORS

Cultural icons. These four stamps, to be released by the U.S. Postal Service in April, honor four American researchers who helped shape science in the 20th century.

DEATHS

Unfinished business. Archaeologist Robson Bonnichsen, a plaintiff in a suit by scientists seeking to study the 9300-year-old remains of Kennewick Man, died in his sleep on Christmas Eve in Bend, Oregon, where he and his wife were visiting family members. He was 64.

Head of the Center for the Study of the First Americans at Texas A&M University in College Station, Bonnichsen never tasted the fruits of the 8-year battle that culminated in victory for the scientists last year (*Science*, 30 July 2004, p. 591). Although the ruling provided for access to the skeleton, which Native American tribes had claimed as an ancestor, the terms are still being negotiated.

"I keep worrying that several plaintiffs are going to be dead before it's decided," his lawyer, Alan Schneider of Portland, Oregon, said prophetically a few years ago. Bonnichsen's death, he now says, "is a shock for all of us."

Doctorate by default. For a young scientist, joining Julius Axelrod's neuroscience lab at the National Institute of Mental Health was once considered a big risk. A chain smoker who spoke with a stutter, Axelrod didn't earn his Ph.D. until his mid 40s, when he bundled together copies of the roughly 100 papers he'd published as a lab technician at NIH and elsewhere. And he didn't act like a scientist: At a time when mentors favored formality, he insisted on being called by his nickname (Julie) by his juniors.

Then in 1970 he won the Nobel Prize for his research on how nerves communicate with one another. And the outsider—blocked from medical school because of Jewish quotas and blind in one eye from an accident in a vitamin-supplement lab in New York City—evolved into a grand old man of science.

Last month Axelrod died at the age of 92. His work revolutionized the field of brain chemistry and led to modern-day treatments for depression and anxiety disorders. He also trained more than 70 scientists. "It's an honor to have been shaped by him," says MIT neuroscientist Richard Wurtman, an early postdoc in Axelrod's lab. "And in my lab, I'm Dick to everyone."

CREDITS (TOP TO BOTTOM): GREG KNOBLOCK/CDC; NIH; TEXAS A&M UNIVERSITY; USPS

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LETTERS

Retraction

IN THE REPORT "LYSOPHOSPHATIDYLCHOLINE as a ligand for the immunoregulatory receptor G2A" by Kabarowski *et al.* (*1*), we concluded that the lysolipid lysophosphatidylcholine (LPC) and a related molecule, sphingosylphosphorylcholine (SPC), directly bound to and served as agonists of the G protein–coupled receptor G2A. Concerns about the reproducibility of portions of the data lead us to retract this paper.

Critical data in the paper showed direct and specific binding of radiolabeled LPC or SPC to G2A in cell homogenates. The primary data generated by Dr. Zhu for these binding studies are not available for evaluation. During investigation of engineered point mutants of the G2A receptor, we were unable to repeat these radiolabeled ligand-binding studies following similar protocols. Alternative protocols with purified membrane fractions (*2*, *3*) expressing high levels of the G2A receptor or whole-cell–based radioligand binding studies (*4*–*6*) also failed to establish direct G2A binding. This calls into question the major conclusion that LPC and SPC are direct ligands for G2A.

In attempts to reproduce LPC stimulation of intracellular calcium responses, only 50% of single MCF 10A cells expressing G2A responded to LPC in single-cell assays identical to those originally employed. Only about half of these gave robust responses similar to those shown in the *Science* paper. Similar assays of intracellular calcium release using bulk cell populations failed to detect any reproducible G2A-mediated response to LPC. Data generated by Dr. Kabarowski demonstrating cellular migration dependent on LPC addition and G2A receptor expression have been reproduced and extended in independent work (*7*–*9*). We believe these data to be accurate and reproducible and therefore conclude that G2A is an effector of LPC action in certain cell-types. However, these data cannot distinguish between a direct action of the lysolipid on the receptor and an indirect action in which the lysolipid modifies another receptor or process that in turn regulates the G2A receptor.

We sincerely regret the confusion that this paper may have caused for the readers of *Science*.

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Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the previous 6 months or issues of general interest. They can be submitted through the Web (www.submit2science.org) or by regular mail (1200 New York Ave., NW, Washington, DC 20005, USA). Letters are not acknowledged upon receipt, nor are authors generally consulted before publication. Whether published in full or in part, letters are subject to editing for clarity and space.

Scientific Priorities in North Korea

IN HIS EDITORIAL "TALKING WITH NORTH Korea" (17 Sept., p. 1677), N. P. Neureiter endorses the idea of scientific cooperation as a tool for engaging the isolated Democratic People's Republic of Korea. This view is echoed by R. Stone in his article "A wary pas de deux" (News Focus, 17 Sept., p. 1696), and each recommends an approach that is both constructive and cautious. We agree but, along with caution, we recommend more urgency to the engagement process. The international and Korean scientific communities should first concentrate on still-widespread food insecurity and a largely dysfunctional health care system before turning its attention to such things as cloning rabbits or breeding supergoats, as mentioned in the article.

Researchers performing embryo transfer on a rabbit in a clean room at the Institute of Experimental Biology in Pyongyang, North Korea.

CREDIT: R. STONE/*SCIENCE*

Throughout the 1990s, North Korea experienced what even its leaders acknowledged was a "march through hardship," including a famine whose most severe years were in 1996 and 1997. Up-to-date, empirical data on mortality were not permitted to be collected inside the country. It became necessary to adopt an indirect approach to data collection, which we did by interviewing a total of 2692 North Korean migrants and asylum seekers who had crossed into China in 1999 to 2000.

In a retrospective household survey of the period 1995–98, we found evidence of elevated crude (all ages, all causes) mortality (peaking at 31.5 per 1000 in 1997), declining fertility, and rising out-migration (*1*, *2*). About 35.8% of deaths (353 of 986) to the 9958 household members during the interval were linked to malnutrition and infectious disease, compared with 11.6% of deaths in 1986 (*3*). A health care system that once produced life expectancies and infant mortality rates comparable to those of South Korea on approximately one-tenth of South Korea's per capita GNP is now overwhelmed by a rising tide of communicable disease, scarce supplies of essential drugs, antiquated equipment, and shortages of heating fuel and electricity in the hospitals and clinics.

In the face of these critical needs, North Korea is increasing some restrictions on foreign aid organizations working inside the country (*4*). Western scientists must join with colleagues in South Korea, China, and elsewhere in Asia to engage with our counterparts in North Korea to promote innovations in the agricultural and health sciences and many other fields, while understanding that North Korean scientists and intellectuals are an elite political class who derive their status and their livelihood from the state. Science to promote state prestige may be different from that which is in the immediate public interest. Engagement must seek to advance science in North Korea for the betterment of all its people.

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Response

I CERTAINLY HAVE NO DISAGREEMENT WITH THE priorities suggested for engagement with

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North Korea by Robinson, Lee, and Burnham. The only problem is that it takes two to tango. I recall that the United States discussed the general idea of exchanges with North Korea at the time of Secretary of State Albright's trip there—the idea was rejected by the North Koreans. The intriguing element of the present initiative is that North Korea has actually proposed the start of some cooperative scientific activity ("A wary pas de deux," R. Stone, News Focus, 17 Sept., p. 1696). If this is real and if they are truly prepared to follow up, I think we should accept this opportunity to begin meaningful cooperation with the North Korean scientific community. Until we have taken a first step toward a cooperative relationship in nonsensitive areas of science, I think it is not useful to try to dictate the priorities for their limited capacity to cooperate with us.

NORMAN P. NEUREITER

AAAS Center for Science, Technology, and Security Policy, 1200 New York Avenue, NW, Washington, DC 20005, USA.

North Korea and Renewable Energy

IN HIS NEWS FOCUS ARTICLE "NUKES FOR windmills: quixotic or serious proposition?" (17 Sept., p. 1698) (and the broader article on North Korean science, "A wary pas de deux," 17 Sept., p. 1696), R. Stone quotes an unofficial envoy of the Democratic People's Republic of Korea (DPRK) as suggesting that the DPRK would be willing to abandon its nuclear program in exchange for clean energy technologies. The desire of North Koreans for renewable small-scale energy systems is consistent with what we have learned in our contacts with DPRK researchers and engineers in the context of our North Korean wind power project (*1*).

The key energy elements of the 1994 Agreed Framework between the United States and the DPRK—the two large (1 GW) light-water reactors (LWRs) and the 500,000 tonnes/year of heavy fuel oil that were to have been provided to the DPRK until the reactors were completed—were political compromises with severe practical drawbacks. The LWRs could not be operated safely without an interconnection to South Korea's grid, and the bottom-of-the-barrel, high-sulfur heavy fuel oil has reportedly accelerated degradation of an already dilapidated thermal power plant fleet (*2*).

Small and mini hydroelectric systems are a good match to the DPRK's terrain and climate, and parts of the DPRK seem to have at least a fair wind resource. Renewable options put the focus on economic redevelopment on the local level, rather than on the less tractable national level.

Renewable energy systems are not going to be enough by themselves to makeover the DPRK's energy sector in the near term, but can certainly contribute to the redevelopment of the DPRK energy infrastructure. They are also relatively resistant to diversion to military use and would engage a broad group of North Korean citizens with visitors from the outside as technological skills are transferred.

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Inflammation and Life-Span

IN THEIR REVIEW "INFLAMMATORY EXPOSURE and historical changes in human life-spans" (17 Sept., p. 1736), C. E. Finch and E. M. Crimmins reinforce earlier suggestions that many diseases and disabilities of older age have their roots in previous exposures to infectious agents and other sources of inflammation in early life. Interesting developments of the inflammatory hypothesis for geriatric illness may come from genetic studies on inflammatory molecules (*1*). Our recent findings allow us to suggest that different alleles at different cytokine genes coding for pro- (IL-6 or IFN-γ) or anti-inflammatory (IL-10) cytokines may affect individual life-span expectancy by influencing the type and intensity of the immune-inflammatory responses against environmental stressors (*2–5*). The conclusion is that people who are genetically predisposed to weak inflammatory activity have a better chance of living longer if they don't catch any infectious diseases.

Our data prompt consideration of the role that antagonistic pleiotropy plays in diseases and in longevity (*6*). Our immune system has evolved to control pathogens, so pro-inflammatory responses are likely to be evolutionarily programmed to resist fatal infections (*7*). Yet genetic backgrounds promoting pro-inflammatory responses play an opposite role in cardiovascular diseases and in longevity (*8–10*), such that cardiovascular diseases are a late consequence of evolutionary programming for a pro-inflammatory response to resist infections at an early age. Genetic polymorphisms responsible for a low inflammatory response may better control inflammatory responses involved in atherogenesis and reduce the risk of atherogenesis complication. So, these polymorphisms might result in an increased chance of long life-span in an environment with reduced antigen (i.e., pathogens) load.

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C. E. FINCH AND E. M. CRIMMINS' REVIEW on the role of reduced inflammation and increased human life-span was most compelling ("Inflammatory exposure and historical changes in human life-spans," 17 Sept., p. 1736). The link between nutrition and inflammation was especially intriguing, especially for those of us involved in Darwinian nutrition issues. With the advent of agriculture, human communities introduced grains, cereals, and other foods whose ratio of omega-6 to omega-3 fatty acids is out of kilter with ancient hominid consumption patterns, a shift that tends to aggravate inflammatory and autoimmune diseases (the pre-agricultural omega-6:omega-3 ratio was approximately 2:1; the ratio in contemporary Americans is as high as 10:1) (*1*). This is being addressed to some degree by food manufacturers and consumer choices, although there is vast room for improve-

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ment. And although it may indeed turn out that, as Finch and Crimmins suggest, "future increases in life expectancy from reduced inflammatory causes may be relatively small," quality of life should be improved considerably as informed populations shift their dietary and life-style patterns to ones that are in harmony with our evolved nature.

I would suggest the elimination of dietary wheat and rye. These grains are especially rich in alkylresorcinols—phenolic lipids that were found to significantly raise thromboxane A2 levels in platelets (*2*). These compounds are absorbed in vivo (*3*). In patients with platelet adherence under way, the release of thromboxane A2 together with ADP can result in the evolution of a platelet thrombus that can lead to a myocardial infarction. Interestingly, it is well established that myocardial infarctions occur most often in the morning hours (*4*–*6*). It is tempting to posit that the inflammation-driven or informed process that underlies thrombus formation may be accelerated by a post-breakfast dietary influx of fats and cereal-derived alkylresorcinols.

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Response

WE AGREE WITH THE DARWINIAN PERSPECTIVES in these Letters, which extend our briefly noted point (p. 1736) that adaptive inflammatory responses to short-term infections can show antagonistic pleiotropy with delayed adverse effects during aging. Payne further notes that diets since the neolithic have increasingly included cultivars containing pro-inflammatory and prothrombotic micronutrients. Of course, these staples were widely used during the 250 years we considered in our Review. It is hard to determine how much of the recent increased longevity is due to improved resistance to infections by consumption of fresh fruit and vegetables year round. However, modern populations show synergistic effects of low levels of antioxidants and high levels of inflammation on old age mortality (*1*).

A further Darwinian question raised by Caruso *et al.* is the role of polymorphisms in genes that influence inflammation and that also show antagonistic pleiotropy. Another example is the apolipoprotein E isoforms (*2*) in which apoE4, the ancestral allele, is associated with elevated cholesterol and can be proinflammatory and prothombotic. The adaptive value of apoE4 during the early reproductive years may depend on the levels of intercurrent infections, such that apoE3, which reduces the risk of dementia, may have become increasingly important to longevity advances as infectious disease waned. Because IL-10 polymorphisms, mentioned by Caruso *et al.*, show evidence of active selection in high disease environments (*3*), one may ask if shifts in inflammatory gene polymorphisms have contributed to the historical changes in longevity.

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HISTORY OF SCIENCE

Lost in Translation?

Stuart McCook

Plants and Empire
Colonial Bioprospecting in the Atlantic World
by Londa Schiebinger

Harvard University Press, Cambridge, MA, 2004. 318 pp. $39.95, £25.95, €36.90. ISBN 0-674-01487-1.

In *Plants and Empire*, Londa Schiebinger uses an innovative analytical approach to revisit the familiar subject of natural history in the colonial Atlantic world. Her study seeks to understand the production of culturally induced scientific ignorance, or agnotology. "Ignorance is often not merely the absence of knowledge," she argues, "but an outcome of cultural and political struggle." In particular, she seeks to understand how and why knowledge of West Indian abortifacients was not transferred to 18th-century Europe. The book explores the history of the silences, struggles, and structures that prevented this transfer.

The 18th-century West Indies were, in Schiebinger's words, a "biocontact zone." The region's inhabitants included people, plants, and animals from the Americas, Africa, and Europe. European bioprospectors scoured the region for new plants and animals of scientific, commercial, or medical value. Schiebinger, a historian of science at Stanford University, paints the 17th and 18th centuries as a period of relative openness in the world of European science. She provides vivid portraits of representative European naturalists, such as the English physician Sir Hans Sloane, who worked in Jamaica, and the Dutch entomologist Maria Sibylla Merian, who worked in Surinam. European naturalists learned much about West Indian flora and fauna from indigenous and African informants, the names of whom are largely lost to history. Such exchanges of information did not take place on an equal footing and were fraught with cultural and social obstacles.

Schiebinger's study explores these exchanges and transfers by focusing on the history of one plant. The peacock flower (*Poinciana pulcherrima*) is a tropical shrub with seeds that have abortifacient properties. Its botanical origins remain obscure, but by the 18th century it was cultivated throughout the West Indies. Amerindian and African communities in the Caribbean had incorporated it into their pharmacopoeia. Schiebinger situates the plant in the context of colonial racial and gender struggles, showing how Africans in particular used abortion as a form of anti-colonial resistance, robbing Europeans of potential labor. Europeans eventually learned about the peacock flower's abortifacient properties. Merian heard about it directly from slave women in Surinam, and she describes its role in slave resistance in her 1704 study of the insects of Surinam. Sloane independently learned about the plant's properties while working as a physician in Jamaica.

The peacock flower itself was first transferred to Europe in the late 17th century. It came to be cultivated in the continent's leading botanical gardens, including the Jardin du Roi in Paris and the Chelsea Physic Garden in London. Schiebinger carefully distinguishes between the transfer of the plant and the transfer of knowledge about the plant. With its flaming red and yellow flowers, *Poinciana* became well known to European gardeners as a favored ornamental. But knowledge of its abortive properties only rarely crossed the Atlantic and did not take root in Europe.

Schiebinger explains this nontransfer of knowledge by situating the peacock flower in the context of 18th-century drug testing and comparing it with similar remedies that were taken up in Europe. During the 18th century, the regulation and systematic testing of drugs became more common. Approved drugs were listed in the official *Pharmacopoeia* of London, Paris, and Amsterdam. Neither the peacock flower nor any other West Indian abortifacient was ever included in 18th-century European pharmacopoeia. Schiebinger shows that this exclusion did not reflect a European prejudice against drugs from the New World: European pharmacopoeias included many New World medicines, such as chinchona to treat malaria and guaiacum to treat syphilis. Nor did it reflect a prejudice against drugs related to women's reproduction. European physicians experimented extensively with emmenagogues—drugs designed to regulate the menses—including many from the New World. Nor were there any official regulations or laws prohibiting the medical study of abortifacients.

The principal obstacle to inclusion was rooted in a broader shift in attitudes toward abortion and abortifacients that took place in the 18th and early 19th centuries. According to Schiebinger, "late eighteenth-century experimental physicians stood at a fork in the road with respect to abortifacients." Abortifacient plants were an integral part of traditional knowledges and practices, in both the Old and New Worlds. Physicians might have chosen to incorporate these plants into their pharmacopoeias, as they did with many other forms of traditional knowledge, or they might have chosen "the road toward the suppression of these knowledges and practices." Almost universally, European physicians chose the latter.

Schiebinger argues carefully that knowledge of the peacock flower and other abortifacients was not overtly suppressed or proscribed. She shows, instead, how the cultural and political structures of 18th-century Europe collectively impeded the transfer of knowledge about abortifacients. She concludes that the "agnotology of abortives among Europeans was not for want of knowledge collected in the colonies; it resulted from protracted struggles over who should control women's fertility." Europe's mercantilist states were anxious to increase their populations, both at home and in the colonies. National wealth and national strength depended on healthy and increasing populations.

Focal flower. Maria Sibylla Merian recorded the use of the *flos pavonis* (peacock flower) to induce abortions in the commentary to this plate (47) from her renowned *Metamorphosis insectorum Surinamensium* (1705).

CREDIT: BILDARCHIV PREUSSISCHER KULTURBESITZ/ART RESOURCE, NY

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Most naturalists and physicians were part of these imperial enterprises to encourage population growth. Even when European naturalists and physicians in the West Indies did learn about new abortifacients, they chose not to disseminate their knowledge. Their counterparts in Europe, similarly, had little incentive to promote the use of abortifacients, or even to study them. Limiting population was simply anathema to the prevailing goals of late 18th-century science and government.

The book does leave some questions unanswered. Religious groups play a central role in contemporary debates over contraception and abortion, so their absence from Schiebinger's account is striking. Some explanation of organized religion's involvement (or non-involvement) in the 18th-century debates would have been helpful. This reservation aside, *Plants and Empire* presents a subtle and compelling explanation for why knowledge of West Indian abortifacients was not taken up by scientists in Europe. More broadly, Schiebinger illustrates the explanatory power of agnotology. Her study of scientific ignorance demonstrates that understanding what scientists do not know is just as important as understanding what they do know.

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MARINE ECOLOGY

Voice of the Turtle

Fredric J. Janzen

"Good will is not turtle soup, but it is an asset all the same." So ends the initial chapter of Archie Carr's seminal book on sea turtles (*1*), which 40 years ago catalyzed global efforts to conserve these charismatic creatures. Aspiring to the same philosophy and influential reach, *Sea Turtles* capitalizes on the depth of James Spotila's experience in field and political environments as well as his evident passion for conservation. These have produced an equally compelling, modern book. Readers of all stripes will be captivated by the outstanding photography and entertained by the stories and descriptions in the book, which admirably bridges the all-too-frequent gap between scientific inquiry and public interest.

Sea Turtles begins with five accessible and thorough chapters on the basic biology of these animals and their relatives. These are followed by individual chapters devoted to each of the seven extant species of sea turtle: green, hawksbill, olive ridley, Kemp's ridley, loggerhead, flatback, and leatherback. Throughout the text, Spotila (a biologist at Drexel University, Philadelphia) frequently sounds the clarion call for conservation of these magnificent animals; that call provides the primary impetus for his offering. He provides fascinating descriptions of human exploitation of turtles and disturbing reports of detrimental ingestion of pollutants by sea turtles of all life stages. But fortunately the book, like Carr's volume, does not skimp on the science or the entertainment. Researchers will find harvestable scientific scholarship in various data-rich tables, such as those that provide precise information on putative genetic factors and incubation temperatures that control offspring sex ratios in sea turtles (which have temperature-dependent sex determination). On the entertainment side, Spotila offers captivating anecdotes of his many personal experiences. (To watch a turtle construct a nest or a neonate hatch from an egg is indeed inspirational.) Even better, he presents a dozen engrossing vignettes of sea turtle "heroes," individuals whose actions have shaped our understanding of these animals or have spurred important conservation efforts.

Dawn departure. A leatherback turtle (*Dermochelys coriacea*) returns to the sea after spending two hours on the beach laying her eggs at Playa Grande, Costa Rica.

CREDIT: EDWARD STANDORA, BUFFALO STATE COLLEGE

Sea Turtles
A Complete Guide to Their Biology, Behavior, and Conservation
by James R. Spotila
Johns Hopkins University Press, Baltimore, MD, 2004. 240 pp. $24.95, £17. ISBN 0-8018-8007-6.

Through his specific pleas for the conservation of sea turtles, Spotila issues a broad challenge to all of us (scientist and layperson alike): if we are to avert the accelerating loss of biodiversity on Earth, we need to take responsibility and get meaningfully involved. In balancing stories of negative human impacts with tales of local conservation successes in this book, the author emerges with an optimistic view of the possible. I certainly hope he is right, but I am less sanguine about the long-term impact of current global conservation efforts. Simply put, humans as a group assign higher priorities to other issues—food, security, personal economics, etc.—that almost invariably conflict with conservation philosophy. And I suspect that most governments will, for economic reasons, side with business interests rather than with the small number of people (albeit passionate and even compelling) who fear for sea turtles and other imperiled organisms. The votes of the conservationists are few, and the environmentally conscious have minimal financial impact on politics compared with those whose livelihoods depend on industries that negatively affect sea turtles and other biota.

Despite the risk of being viewed a Cassandra, I suspect that only the experience of a biological catastrophe on the order of a major economic upheaval will compel human societies to respond with appropriate alacrity to the alarming global destruction of biota that we scientists are documenting. You can convince yourself by considering this question: Does the extinction of, say, the Colombian grebe effectively mean anything to the average commuter who travels an hour or more each way to a job in a big city, to the hardworking immigrant who processes cattle in a meatpacking plant in the Great Plains, or to the typical individual who furnishes a house with objects fashioned from hardwood imported from the tropics? The answer in this one instance is clearly "no." Yet it is remarkable that the recent, human-induced loss of orders of magnitude of Earth's biodiversity through the cumulative effect of such individual extinctions has not elicited a public outcry sufficiently powerful to initiate meaningful change. In this view, the numerous well-meaning conservation efforts around the globe—including those advocated by Spotila and undertaken by many (myself included)—are merely nibbling at the edges of the ultimate problem and simply delaying the inevitable. And worse, as Spotila rightly points out, the long lives of many organisms such as turtles mask their ongoing declines. By the time the catastrophic demise of these species is first noticed by the public, it is often too late to restore them, much less maintain their genetic integrity (*2*).

Earth's current biodiversity crisis aside, Spotila provides a wonderful entrée into the exciting world of sea turtles for the uninitiated and a delightful repast for everyone. His eloquent words are inspiring, and his hopeful message deserves to be heard by a broad audience. May we and countless generations of our descendants always hear the voice of the turtle.

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ECOLOGY

The Convention on Biological Diversity's 2010 Target

Andrew Balmford, Leon Bennun, Ben ten Brink, David Cooper, Isabelle M. Côté, Peter Crane, Andrew Dobson,* Nigel Dudley, Ian Dutton, Rhys E. Green, Richard D. Gregory, Jeremy Harrison, Elizabeth T. Kennedy, Claire Kremen, Nigel Leader-Williams, Thomas E. Lovejoy, Georgina Mace, Robert May, Phillipe Mayaux, Paul Morling, Joanna Phillips, Kent Redford, Taylor H. Ricketts, Jon Paul Rodríguez, M. Sanjayan, Peter J. Schei, Albert S. van Jaarsveld, Bruno A. Walther

Most of the time, most of us behave as if our ongoing destruction of biological diversity and natural ecosystems has a net beneficial effect on our personal well-being. This is because it often has—locally, in the short term, and for people with the most power. However, when a longer-term view is taken, conserving biodiversity and the services it provides emerges as essential to human self-interest (*1*, *2*). Representatives of 190 countries at the 2002 Johannesburg World Summit on Sustainable Development committed themselves to "…achieving by 2010 a significant reduction of the current rate of biodiversity loss at the global, regional, and national level…" (*3*). By adopting the 2010 target, governments are explicitly recognizing the value of biodiversity, setting goals for its conservation, and holding themselves accountable (*4*, *5*).

These undertakings present conservation scientists with a great challenge. The 2010 target can only catalyze effective conservation if systems are in place to tell governments, businesses, and individuals about the consequences of their actions. Yet we have so far identified only a fraction of the earth's biological diversity and have just a rudimentary understanding of how biological, geophysical, and geochemical processes interact to contribute to human well-being. How can we present our knowledge in ways that are useful to decision-makers and in time to contribute to achieving the 2010 target?

The Need for Indicators

Part of the answer lies in establishment of indicators of biodiversity and ecosystem functions and services that are rigorous, repeatable, widely accepted, and easily understood. Conservation scientists have a lot to learn in this regard from economists, who have long had a set of common and clear indicators for tracking and influencing market development. Recently, biologists adopted a similar approach by producing composite indicators from population time series data on widely studied groups such as birds and other vertebrates (*3*, *6*–*10*). One of these, the U.K. Wild Bird Index, has already been adopted by the U.K. government as an indicator of quality of life and a measure of how well environmental policies are working (*6*, *11*); because of well-understood links with farming practices (*12*), this index could soon be extended to the European Union (EU) to inform the reshaping of its Common Agricultural Policy (*6*).

The first step toward developing global indicators has already been taken. In early 2004, parties to the Convention on Biological Diversity (CBD) established a framework for assessing progress on the 2010 target [United Nations Environment Programme (UNEP) (*13*); see table, p. 213]. For these indicators to gain wider scientific respect and be used more broadly, they will require continuing independent scientific assessment and input. In July 2004, the Royal Society (U.K.) invited more than 60 scientists from governments, academia, and global and national conservation organizations (representing 15 countries) to a workshop designed to review the indicators and to explore how such input could be provided.

Workshop participants concluded that the 18 indicators already identified are likely to provide useful information but also will leave important gaps in our understanding of biodiversity loss. Additional indicators were proposed that could provide some of the missing information by 2010. A comprehensive set of indicators may need to be larger still [e.g., see 102 indicators for taking the pulse of U.S. ecosystems (*14*)]. However, workshop participants recognized that developing indicators would not be enough.

Broadening the Science

Fundamentally, we need to develop models that describe how the human, biological, physical, and chemical components of the earth system interact. Sketching the scope of such models (see SOM) brings home the fact that while we have little detailed and quantitative information on many components of the system, we know even less about how the linkages between them work. Developing models would guide data collection, help quantify how ecosystems benefit humans, clarify mechanisms by which activities and policies affect biodiversity and the services it provides, and allow improved projections about what might happen in the future. Part of the work of the Millennium Ecosystem Assessment (*15*) is to build models of this kind, but this effort needs to be continued and extended.

Most of the indicators so far under discussion deal with biodiversity per se and principally involve biologists. Studies linking socio-economic factors and geophysical and geochemical processes with biodiversity are relatively undeveloped. Given the contributions that biodiversity conservation will make toward alleviating poverty (*16*, *17*), it is crucial that indicators and models address all components.

Reducing the rate of loss of a plant or animal species is only a step in the right direction and may not prevent extinction. Likewise, preventing further decline and even allowing modest recovery, for example, of a depleted fish stock, might not be sufficient to allow sustainable exploitation (*18*). Policy-makers may need to consider more ambitious targets, such as halting loss and restoring ecosystems. This was already accepted by the EU Council at its meeting in Göteborg, Sweden, in 2001 and by the European Environment Ministers at Kiev, Ukraine, in 2003 (*19*).

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There are also immediate needs for global extension of monitoring programs and developments in capacity building, design of data collection programs, quality control, and statistical analyses. Most indicators likely to be available in the near future will be based on existing databases and monitoring schemes. However, as the areas richest in biological diversity are often those most lacking resources, current databases and monitoring are usually not fully representative and do not cover a wide enough range of system components. Meta-analyses of other existing, if scattered, data offer considerable scope for plugging some gaps quickly (*20*). Another possibility is the use of remote sensing to measure both currently and retrospectively the extent and condition of biomes. This approach is already well developed for measuring changes globally in forests (*21*).

The Challenge

The 2010 target provides the scientific community the challenge to engage in exciting fundamental science and to participate in what is likely to be the most significant conservation agreement of the early 21st century. Models, indicators, data, and monitoring techniques must be open to scrutiny. Interdisciplinary collaboration will be essential to strengthen the scientific rigor of the indicators, to enhance their relevance to policy, and to raise public awareness of their usefulness. Scientists must act in four key ways: (i) work with the CBD Secretariat and its partners to develop, review, and use the indicators already identified by the CBD Conference of Parties (*22*); (ii) develop research and monitoring programs; (iii) share information and experience regarding development and implementation of monitoring programs, data management, and sharing; and (iv) promote increased availability of funds for long-term research and monitoring programs.

Economic indicators like gross domestic product (GDP) and financial indicators like the Dow Jones have set the precedent. The global imperative to protect biodiversity and ecosystem services must become as politically significant as economic growth, and the reasons for reducing the rate of loss of biological diversity need to be as widely understood and valued by the public and by governments. Well-conceived, robust, and understandable indicators can help achieve this objective. Yet time is fast running out: We are already approaching the half-way mark of this extraordinary chance for global conservation.

CONVENTION ON BIOLOGICAL DIVERSITY'S FRAMEWORK FOR ASSESSMENT BY 2010

Identified indicators	Proposed indicators
Components of biological diversity	
• **Forest area**	• Condition of forests
• **Trends in abundance and distribution of selected species**	• Extent and condition of shrublands, grasslands, and deserts
• **Coverage of protected areas**	• Extent of wetlands and large water bodies
• Change in status of threatened species	• Catchment condition—extent of riparian vegetation
• Trends in genetic diversity of domesticated plants and animals	• Percent live coral cover
• Extent and location of mangroves and seagrass and macroalgal beds	• Extent and condition of estuaries
• Management effectiveness of protected areas	
• Investment in protected areas	
Sustainable use	
• Area of forest, agriculture, and aquaculture under sustainable management	
• Proportion of products derived from sustainable sources	
Threats to biodiversity	
• **Nitrogen deposition**	• Marine fishing effort
• Number and cost of alien invasions	• Road-free area
	• Epidemic outbreaks among wild species
Ecosystem integrity, goods, and services	
• **Marine trophic index**	• Number of dams
• **Water quality in inland waters**	• Sediment load in rivers
• Freshwater trophic index	• Percent population without potable water
• Connectivity and fragmentation of ecosystems	• Carbon storage in ecosystems
• Incidence of human-induced ecosystem failure	• Market share of nature-based tourism
• Health and well-being of people in biodiversity-dependent communities	• Hit rates for biodiversity-related website
• Biodiversity use in food and medicine	• Pesticide use per unit agricultural harvest
• Fish harvest per unit effort	• Agricultural harvest per unit effort
• Timber and fuelwood harvest per unit effort	
Traditional knowledge, innovations, and practices	
• **Status and trends of linguistic diversity and numbers of speakers of indigenous languages**	
Resource transfers	
• **Official development assistance in support of CBD**	

The CBD framework for assessing progress. The 18 indicators already identified for immediate testing (bold) and future development (not bold) are shown plus indicators suggested by the Royal Society workshop and potentially available by 2010. Workshop recommendations can be viewed at www.twentyten.net.

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Supporting Online Material

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MICROBIOLOGY

TB—A New Target, a New Drug

Stewart T. Cole and Pedro M. Alzari

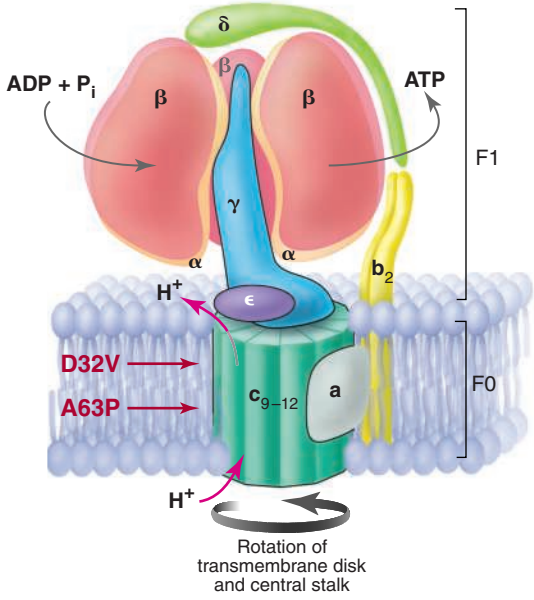
Although the outbreaks of multidrug-resistant tuberculosis (MDR-TB) that afflicted New York City in the 1990s were relatively minor when compared to the burden of global tuberculosis (*1*, *2*), they served to raise public and political awareness. The result was that for the first time TB control was included on the agenda of the G8 economic summit meetings. The world's leaders lent their support to that of nongovernmental organizations, such as the Global Alliance for TB Drug Development (GATDD) and the World Health Organization (WHO), by encouraging industry and academia to engage in the development of new drugs to treat this chronic respiratory disease. This was a crucial event given that TB claims up to 2 million lives annually worldwide, blights myriad communities principally in developing countries, and that no new TB drugs have been discovered in the past 40 years (*2*). Regarding new drugs to combat TB, good news is reported by Andries *et al.* (*3*) on page 223 of this issue. These investigators identify a highly active new TB drug that will provide a welcome boost to TB patients, physicians, and health care workers, as well as the pharmaceutical industry. The pharmaceutical industry has singularly failed to produce adequate new anti-infective agents in the past decade despite access to high-throughput screening facilities, enormous chemical libraries, and structure-assisted drug design.

Enhanced online at www.sciencemag.org/cgi/content/full/307/5707/214

The current treatment for TB recommended by WHO—known as directly observed therapy short-course (DOTS)—requires patients to adhere to a three- or four-drug regimen comprising isoniazid, rifampin, pyrazinamide, and/or ethambutol for a minimum of 6 months. All of these drugs are old and unattractive by today's standards. Many patients fail to complete therapy because of drug side effects and the complicated drug regimen, resulting in relapse—often in the form of MDR-TB, which is even more difficult to treat. In an authoritative review (*4*), the GATDD identified several means of improving therapy: An ideal new TB drug should be highly active, so that treatment duration can be reduced to <3 months; it should kill the persistent bacilli that might otherwise reactivate later in life; and it must show activity against MDR-TB strains. Optimally, a new therapeutic agent would be specific for *Mycobacterium tuberculosis* and also compatible with existing TB drugs, because combination therapy will remain mandatory to combat this major killer.

The new candidate drug (*3*) developed by the Johnson and Johnson group and reported in this issue (*3*) meets nearly all of these criteria. Its lead compound was identified by adopting a medium-throughput screening approach using live mycobacteria rather than the more popular target-based, high-throughput screening that uses robotics to screen millions of compounds for inhibitors of critical functions such as key enzyme activities. This proved a very astute decision because it avoided problems with drug permeability (which always affect the target-based screens at a later stage) by identifying active compounds that freely entered the mycobacteria. The elegant strategy of Andries *et al.* revealed a new class of inhibitor that blocks the function of an essential membrane-bound enzyme that makes adenosine triphosphate (ATP). Such inhibitors would have been less likely to be discovered by more traditional approaches. Each of the "hits" in the medium-throughput screen belonged to the diarylquinoline family of chemical compounds. After optimization by synthetic chemistry, the investigators were left with 20 interesting drug candidates; of these, R207910 showed the best activity profile. R207910 is bactericidal and exquisitely active against a broad range of mycobacteria, displaying little or no activity against the other microorganisms tested. Crucially, R207910 is active against both the drug-sensitive and drug-resistant forms of *M. tuberculosis.* This organic compound of 555.51 daltons, which contains both planar hydrophobic moieties and hydrogen-bonding acceptor and donor groups, displays perfect drug-like features that satisfy most of Lipinski's rules for good drug candidates (*5*). Pharmacokinetic and pharmacodynamic studies in different animal models have confirmed the excellent drug-like properties of diarylquinolines.

To identify the target of R207910, Andries *et al.* isolated mutants of *M. tuberculosis* and the related faster-growing organism *M. smegmatis* that were resistant to R207910, and characterized them by whole-genome sequencing. They then identified two different missense mutations in the *atpE* gene, which encodes the C subunit of ATP synthase, the enzyme that uses the transmembrane proton-motive force to generate ATP for the



A chink in the mycobacterial armor. Model of the mycobacterial ATP synthase showing the position of mutations that confer resistance to the diarylquinoline drug R207910 (*3*). ATP synthase has two major structural domains, F0 and F1, that act as a biological rotary motor (*6*). F1 is composed of nine subunits (α3, β3, γ, δ, ε) and is located in the cytoplasm, where it generates ATP. F0 spans the cytoplasmic membrane and contains 13 to 15 subunits (a, b_2, c_{9-12}) arranged as a symmetrical disc. The F0 and F1 domains are linked by subunits γ, ε, δ, and b_2. Rotation of the transmembrane disc and the central stalk is driven by the proton-motive force. The c subunit is an α-helical hairpin structure with a short connecting loop. Both mutations associated with R207910 resistance affect these membrane-spanning α helices. Notably, the A63P mutation is very near E60, the glutamic acid residue whose carboxyl group is protonated during proton translocation.

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cell (*6*). ATP synthase is a biological rotary motor made up of two major structural domains, F0 and F1 (see the figure). The F1 domain is composed of subunits α_3, β_3, γ, δ, and ε; the F0 domain includes one a subunit, two b subunits, and 9 to 12 c subunits arranged in a symmetrical disk. The F0 and F1 domains are linked by central stalks (subunits γ and ε) and peripheral stalks (subunits b and δ). The proton-motive force fuels the rotation of the transmembrane disk and the central stalk, which in turn modulates the nucleotide affinity in the catalytic β subunit, leading to the production of ATP. The c subunit has a hairpin structure with two α helices and a short connecting loop. The two mutations affect the membrane-spanning α helices of the ATP synthase c subunit and may restrict binding of R207910 to the enzyme. Although biochemical confirmation is now required, it is possible that the drug impedes assembly of the mobile disk or interferes with its rotational properties, leading to inadequate synthesis of ATP.

A puzzling feature of R207910 is its exceptional specificity for mycobacteria. ATP synthase is a ubiquitous enzyme found in most living organisms, including humans. There is very limited sequence similarity between the mycobacterial and human AtpE proteins, which bodes well for the safety of the compound, as borne out by the phase I study in human volunteers. The mycobacteria-specific activity of R207910 [(*3*), table S1] may also be the consequence of limited sequence similarity among bacterial AtpE proteins. However, those antitubercular agents that show highly restricted activity (such as isoniazid, ethionamide, and pyrazinamide) are all prodrugs requiring activation by a mycobacterial enzyme (*7*). Although its chemical structure gives no clues to potential activation sites, R207910 may also prove to be a prodrug.

The discovery of R207910 will generate considerable excitement and optimism among all those involved in the treatment and management of tuberculosis. Mouse studies already show that this compound can greatly shorten the duration of therapy, both alone and in association with current antitubercular agents. The DNA gyrase inhibitor moxifloxacin has recently shown similar promise in the same animal models (*8*). For the first time in many years, there is real hope of achieving the quantum therapeutic leap required to make an impact on the global TB epidemic. It is therefore of the utmost importance that R207910 should now enter phase II clinical trials. Furthermore, the equally remarkable activity of R207910 against *M. ulcerans*—the agent of an emerging human disease called Buruli ulcer (*9*), for which surgery is the only cure—also raises expectations for a safer treatment for this disfiguring affliction.

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APPLIED PHYSICS

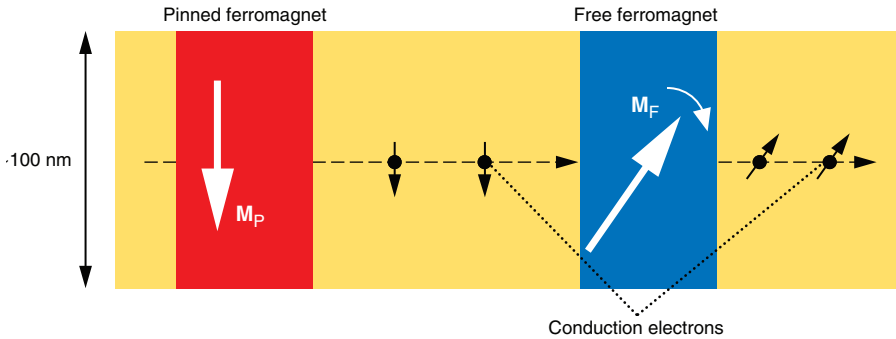
A Ringing Confirmation of Spintronics Theory

Mark Covington

Electrons possess both electric charge and angular momentum (or spin). Traditional electronic devices use only charge, but a growing class of electronic devices exploits spin. One example is the spin-dependent magnetoresistive readback sensors used in hard disk drives and in emerging nonvolatile magnetic memories. However, even more ways to use spin are being proposed for new spin-based electronics, or "spintronics" (*1*).

It has been shown that a current of spin-polarized electrons can change the magnetic orientation of a nanometer-scale ferromagnet via an exchange of spin angular momentum (*2*, *3*). This effect originates from the way in which ferromagnets align the spin of conduction electrons along the direction of magnetization. In other words, ferromagnets exert a torque that changes the electron angular momentum. Conversely, conservation of angular momentum requires a back-action torque on the magnet. Theory predicts that this torque differs fundamentally from the usual torque exerted by magnetic fields. The most direct way to test this prediction experimentally is to study the dynamical motion of a nanomagnet in response to a spin-polarized current. On page 228 of this issue, Krivorotov *et al.* (*4*) present an extensive set of dynamical measurements that elucidate this effect (see the first figure).

How does a nanomagnet respond to spin transfer? The relative orientation of the electron spins and the magnet determines whether the spin torque augments or opposes the damping torque that forces the magnet to settle into static equilibrium. Within this scenario, two competing models predict very distinct behavior when spin transfer reverses, or switches, a nanomagnet. The spin-torque model predicts that nanomagnets respond coherently to spin-polarized electrons (*3*). Depending on the strength of the spin torque relative to the damping, three different types of dynamical states can



Schematic of the "nanopillar" structure used by Krivorotov *et al.* (*4*). Electrons polarized by the pinned ferromagnet exert a torque on the free ferromagnet. At these nanoscale dimensions, spin transfer dominates over the magnetic field produced by the moving electrons, and the large current densities that are necessary to induce a response are easily achieved. Motion of the free layer magnetization, $\mathbf{M}_F$, is monitored through the resistance, which depends on the relative orientation of $\mathbf{M}_F$ and the pinned-layer magnetization, $\mathbf{M}_P$. The resistance continuously varies from low to high resistance as $\mathbf{M}_F$ and $\mathbf{M}_P$ go from parallel to antiparallel, respectively.

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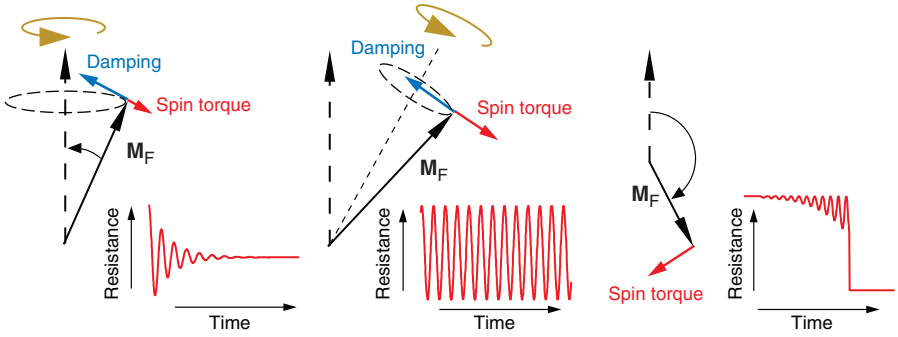
occur (see the second figure). Reversal occurs through spatially and temporally coherent precession of the magnetization. Another model proposes that spin transfer induces incoherent, short-wavelength magnetic oscillations that mimic what would happen if the magnet got hot (*5*, *6*). The magnetization then switches in a stochastic manner akin to a thermally activated process.

The experiments of Krivorotov *et al.* provide direct evidence for the coherent switching process predicted by the spin-torque model. When a sufficiently large current pulse is sent through a nanomagnet, such that the spin torque opposes the damping, the forces that keep the magnet settled along a particular direction are overcome, and the magnet starts to rotate in response to the driving torque from the electrons. The electrons continually impart angular momentum to the magnetization before the precession has a chance to die down. The amplitude of this oscillation, or "ringing," increases until the magnet reverses its direction (see the second figure, right panel). Larger currents drive this switching process even faster. This is what Krivorotov *et al.* observe experimentally.

Spin transfer also affects the magnetization dynamics below the switching threshold. Situations can occur where the spin torque effectively counterbalances damping. In this case, the magnet neither switches nor settles back into equilibrium but instead rings indefinitely (see the second figure, middle panel). Hence, a dc current can drive microwave oscillations, which can potentially be used as microwave source.

Krivorotov *et al.* observe this steady-state precession, confirming previous measurements (*7*–*9*). Moreover, they show that the magnetic precession is synchronous with the current pulse and can quickly wind up to its full amplitude in only a few periods (less than 1×10^{-9} s). Finally, they demonstrate that a dc current can affect the time it takes for the magnetization to settle into static equilibrium (see the second figure, left panel). These data provide clear proof of the spin-torque model by demonstrating that spin transfer can continuously tune the magnetic damping and induce coherent magnetic motion.

The precise, deterministic magnetic motion induced by spin transfer is already being explored for use as tunable magnetic-based microwave oscillators in logic and communications applications (*8*). Magnetic memory is another application for which spin transfer seems well suited. In addition to its ability to switch a magnet between bistable states (that is, either a "0" and a "1"), switching with spin transfer is more efficient than with magnetic fields at nanoscale dimensions. Because miniaturization is required to achieve higher performance and lower cost in solid-state electronics, spin transfer has the potential to replace field-driven switching in magnetic memory and enable ever higher storage capacity.



Dynamical regimes where spin transfer opposes damping. (**Left**) When the spin torque is smaller than the damping torque, precession is quickly damped and the magnet settles into static equilibrium (dashed arrow). The time scale of the damping can be tuned continuously by the current. (**Middle**) When the spin torque and the damping torque are effectively equal and opposite over a precessional orbit, persistent precession occurs. (**Right**) When the spin torque is larger than the damping torque, the precession increases in amplitude until the magnetization completely reverses direction.

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10.1126/science.1107610

CHEMISTRY

Odd Electron on Nitrogen: A Metal-Stabilized Aminyl Radical

Wolfgang Kaim

Carbon- and oxygen-centered organic radicals were once considered chemical curiosities or, at best, reactive intermediates. However, in recent years some of these molecules have received widespread attention beyond chemistry—for example, as spin carriers in materials science (*1*) or as reaction sites in biology (*2*–*7*). Stable organic radicals with the unpaired ("odd") electron centered on nitrogen have received less attention, although some examples have been known since the late 19th century. On page 235 of this issue, Büttner *et al.* (*8*) report the first isolation and unambiguous characterization of an aminyl ($NR_2^{\bullet}$) radical stabilized by metal coordination.

Radical cations of nitrogen-containing amino acids such as tryptophan or histidine have recently been discussed in connection with electron transfer in cytochrome c peroxidase (*6*) and photosystem II of photosynthesis (*7*). Aminyl radicals ($NR_2^{\bullet}$, where R is an aryl or alkyl) are the most elementary class of nitrogen-centered organic radicals. An earlier report of their stabilization through metal coordination was proven erroneous because of intramolecular reduction to an amide (NR_2^-) ligand (*9*, *10*). After that false start, Büttner *et al*. now demonstrate (*8*) that an aminyl radical can indeed be stabilized by metal coordination.

The chemical properties of aminyl radicals are intermediate between those of alkyl radicals ($CR_3^{\bullet}$) and aryloxy species ($OR^{\bullet}$ with R = aryl). Alkyl radicals have essential biochemical roles, for example as $CH_2R^{\bullet}$ in coenzyme B_{12}–dependent processes (see the figure, top left panel) (*3*). Aryloxy species also have established functions in oxidation reactions (see the figure, bottom right panel) (*4*, *5*), photosynthesis (*7*), and DNA synthesis (*6*). In almost all cases, the radicals are accompanied by transition metal ions, which can activate and control these reactive species through electron transfer. Aminyl radicals

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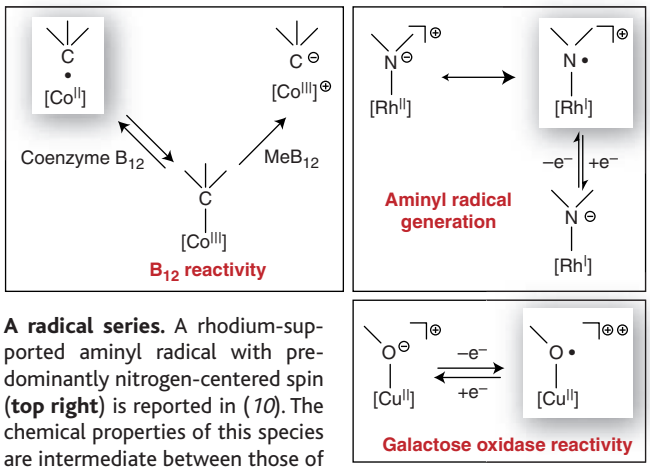
have not yet been implicated in biochemical processes, even though amines play many roles in metabolism, information transfer, and enzymatic activity.

Büttner *et al.* (*8*) were able to stabilize an aminyl radical by using large R groups in $NR_2^{\bullet}$ and binding it to an electronically active (*11*) rhodium complex (see the figure, top right panel). They use crystallographic data, quantum chemical calculations, and sophisticated electron paramagnetic resonance measurements to establish the location of the unpaired electron. According to the calculations, 57% of this electron's spin resides on the aminyl nitrogen atom. Preliminary reactivity studies reveal that the radical has a nucleophilic character (*8*).



A radical series. A rhodium-supported aminyl radical with predominantly nitrogen-centered spin (**top right**) is reported in (*10*). The chemical properties of this species are intermediate between those of metal-interacting carbon- and oxygen-centered radicals, which are well-established in biochemistry. For example, carbon-centered radicals play a role in coenzyme B_{12} reactivity (**top left**), and oxygen-centered radicals interact with copper in the alcohol-oxidizing galactose oxidase (**right**) (*4*).

Radicals combined with electron transfer–active transition metals can serve a range of functions (see the figure). In the coenzyme B_{12}, the cobalt atom is responsible for the reversible generation and scavenging of alkyl radicals (*3*). In copper-dependent galactose oxidase and related enzymes, the metal plays a more extensive role: The metal binds O_2 and the substrate, and a modified tyrosinate/tyrosyl radical couple acts as an additional electron transfer site. High metal oxidation states such as Cu(III) can thus be avoided (*5*). A similar role is played by the tyrosyl radicals in ribonucleotide reductases (*6*) and photosystem II (*7*). In the case of aminyl radicals, the binding of aminyl to a spin-paired transition metal of relatively low oxidation state could be a precursor for an effective radical nucleophile reaction site.

Metal-associated inorganic ($O_2^{\bullet-}$, $NO^{\bullet}$) and organic radicals are ubiquitous and have diverse biological functions (*2*–*7*, *12*). On the other hand, amines are known to have various physiological roles. The report of a synthetic, metal-stabilized aminyl radical by Büttner *et al.* (*8*) suggests that metal-associated aminyl radicals are likely to be discovered in a biological context in the next few years.

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CELL SIGNALING

Stat Acetylation—A Key Facet of Cytokine Signaling?

John J. O'Shea, Yuka Kanno, Xiaomin Chen, David E. Levy

DNA binding proteins called Stats (signal transducers and activators of transcription) carry signals from cytokines and other extracellular stimuli into the cell nucleus (*1*, *2*). Stats each carry a Src homology 2 (SH2) domain that recruits Stats to cytokine receptors at the cell surface that have been stimulated by ligand and phosphorylated. Here, Stat dimers form through association of their two phosphorylated SH2 domains, and then move to the nucleus where they bind to the DNA and switch on the expression of specific target genes.

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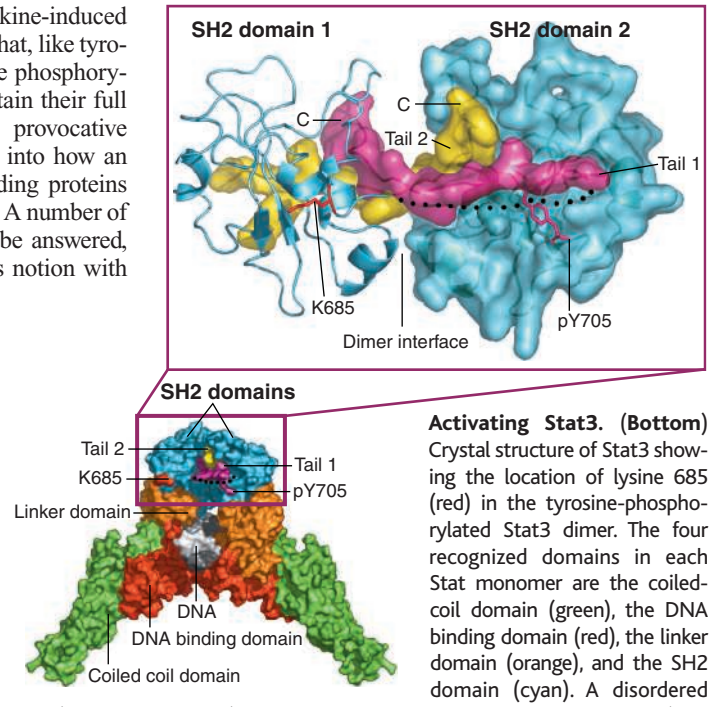
Some Stats are also phosphorylated on a conserved serine amino acid residue in the transcriptional-activation domain of their carboxyl terminus (*3*). Although phosphorlation is a crucial posttranslational mechanism that regulates the activities of numerous proteins, there are many others including methylation, ubiquitination, sumoylation, isgylation, and acetylation. Indeed, different Stat proteins undergo different modifications, yielding a variety of consequences for the transcription of target genes. On page 269 of this issue, Yuan *et al.* (*4*) report that Stats undergo acetylation of a single amino acid residue, lysine 685, in response to cytokine stimulation. They conclude that this modification is essential for Stats to form stable dimers and to activate transcription.

Using an antibody that detects lysine acetylation, Yuan *et al.* demonstrated that cytokine stimulation induced acetylation of Stat3 in the cytosol of cultured cells. When cells overexpressed p300 or CBP—histone acetyltransferase (HAT) enzymes that add acetyl groups to amino acids—Stat3 became acetylated. Meanwhile, overexpression of histone deacetylase (HDAC) 1, 2, or 3 (which remove acetyl groups from amino acids) reduced the acetylation of Stat3. Cytokine stimulation of cultured cells promoted the association of p300 with Stat3, whereas reducing p300 levels attenuated activation of transcription from a Stat reporter construct. In addition, overexpression of histone deacetylase impaired Stat-mediated activation of transcription from the reporter. Histone acetylases associated with Stat3 after ligand stimulation, but the association of histone deacetylases with Stat3 was variable and only partially modulated by cytokine stimulation.

By mutating lysine residues in Stat3, the investigators found that lysine 685 (an amino acid conserved in several Stats) was required for Stat3 acetylation. Mutant Stat3 carrying an arginine instead of a lysine residue at position 685 could be phosphorylated on tyrosine and serine and did move to the nucleus, but its ability to form dimers and to bind to DNA was impaired. Functionally, this mutant Stat did not mediate cytokine-dependent induction of gene expression or enable cellular proliferation. The authors conclude that

acetylation of Stats is a cytokine-induced posttranslational modification that, like tyrosine phosphorylation and serine phosphorylation, is critical for Stats to attain their full transcriptional potential. This provocative finding provides fresh insights into how an important family of DNA binding proteins might regulate gene expression. A number of questions, however, remain to be answered, including how to reconcile this notion with previous findings.

First, based on the crystal structure of Stat3, it is hard to conceive how the putative acetylated lysine in Stat3 could be directly involved in the formation of Stat dimers. Lysine 685 in Stat3 is situated on the external surface of the SH2 domain with its side chain exposed (see the figure). Although lysine 685 is near the phospho-Stat dimer interface, it seems to have no structural role in mediating dimer formation. An important concern of the Yuan *et al*. study is that mutation of lysine 685 to an arginine could have disrupted the structure of Stat in a manner unrelated to acetylation.



Activating Stat3. (**Bottom**) Crystal structure of Stat3 showing the location of lysine 685 (red) in the tyrosine-phosphorylated Stat3 dimer. The four recognized domains in each Stat monomer are the coiled-coil domain (green), the DNA binding domain (red), the linker domain (orange), and the SH2 domain (cyan). A disordered loop (black dotted curve) connects the SH2 domain on the left to Tail 1 (magenta). Two strands of DNA are shown in white and black. The other lysine 685 is on the back side of the SH2 domain on the right. (**Top**) Detailed view of the dimer interface of the two tyrosine-phosphorylated SH2 domains (cyan) of Stat3. Two tail segments are shown in magenta and yellow, and the dotted curve is a disordered loop (residues 689 to 701) connecting the SH2 domain on the left to its tail segment. Lysine 685 is depicted in red and tyrosine 705 is shown in magenta. [Adapted from (*14*) using PyMOL]

Second, the involvement of histone acetylases and deacetylases in the regulation of cytokine signaling is far from simple. Recent data point to a positive role for histone deacetylases in cytokine-dependent gene regulation, not the negative role implied here. Previous reports have been ambiguous concerning the effect of Stat acetylation on transcriptional activity (*5*–*7*). Yet more recent data have shown that Stat responses are blocked, not enhanced, by inhibition or reduced expression of histone deacetylase (*8*–*11*). A caveat of the new work is that it relies heavily on overexpression to establish most of its major conclusions (*4*). Furthermore, experiments using reporter constructs must be viewed with caution, given the very variable effects produced by overexpression of histone acetyltransferase and histone deacetylase. Indeed, the Yuan *et al.* findings are open to alternative explanations. For example, the effects of histone acetyltransferase, especially in reporter assays, may not be due to Stat3 acetylation per se but instead may be due to effects on other processes.

The data pertaining to the interactions of Stat3 with histone acetyltransferase or histone deacetylase are also puzzling. The authors demonstrate the association of p300 with Stats and the dissociation of HDAC3 from Stat3 after ligand stimulation of cultured cells. Because histone acetyltransferase and histone deacetylase proteins are usually found in the cell nucleus in distinct multimeric protein complexes, it is difficult to know whether all of these interactions are physiologically meaningful. Also, it is difficult to envision the intracellular compartments that house interactions between Stat3 and either histone acetyltransferase or histone deacetylase, and how these interactions are regulated by cytokines. What is the signal and how does the engaged receptor activate this process? Are the Jaks (Janus kinases) involved?

The nuclear transcription factor NF-κB (RelA) is also acetylated by p300 and deacetylated by HDAC3 in a signal-dependent manner (*12*, *13*). In this case, however, the details seem clearer as deacetylation is specifically mediated by HDAC3, not HDAC1. Acetylation of NF-κB prevents this transcription factor from interacting with its inhibitor IκB, resulting in lifting of NF-κB repression and prolonged expression of target genes. Meanwhile, deacetylation promotes assembly of NF-κB with IκB, resulting in decreased expression of target genes. The signal dependence of acetylation appears to be linked to nuclear translocation. Shuttling of NF-κB into the nucleus brings this transcription factor close to nuclear acetyltransferases, thus facilitating its acetylation, providing a mechanistic basic for the effect of cytokines. A similar understanding of Stat3 modification is difficult to reconcile with the finding that Stat3 acetylation and deacetylation occur in the cytoplasm as well as the nucleus and can be mediated by multiple enzymes.

Clearly, much work remains to be done before we can understand the cell biology and biochemistry of Stat acetylation. Defining the mechanisms by which cytokine stimulation controls Stat acetylation and how acetylation regulates Stat function will be key to this understanding. Perhaps some of the murkiness surrounding the new findings is due to the complexity of protein modifications. For example, posttranslational modifications may be mutually exclusive, such that lysine acetylation could prevent an alternative modification that shuts down Stat activity, resulting in a net positive effect. One very important piece of information currently missing is the stoichiometry of these modifications. Although tyrosine phosphorylation of Stats tracks absolutely with dimer-induced activity, similar data for acetylation are lacking. Defining how histone acetyltransferases, histone deacetylases, and other modifying enzymes are involved in cytokine responses and sorting out their substrates will require considerable effort. Reconciling the Yuan *et al*. results with recent reports of the positive effects of histone deacetylases on transcriptional activity will be key to clarifying this important area of cytokine signaling.

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Testing Hypotheses: Prediction and Prejudice

Peter Lipton

Observations that fit a hypothesis may be made before or after the hypothesis is formulated. Can that difference be relevant to the amount of support that the observations provide for the hypothesis? Philosophers of science and statisticians are both divided on this question, but there is an argument that predictions ought to count more than accommodations, because of the risk of "fudging" that accommodations run and predictions avoid.

In the case of "accommodation," a hypothesis is constructed to fit an observation that has already been made. In the case of "prediction," the hypothesis, though it may already be partially based on an existing data set, is formulated before the empirical claim in question is deduced and verified by observation. Well-supported hypotheses often have both accommodations and successful predictions to their credit. Most people, however, appear to be more impressed by predictions than by accommodations. Edmond Halley was able to account for the observed comets of 1531, 1607, and 1682 as a single object with a perturbed elliptical orbit. Natural philosophers took some notice when he published his views in the *Philosophical Transactions* in 1705, but it was only when his prediction of the return of the comet in 1758 was confirmed that the entire European intellectual world embraced Halley's Comet. The single prediction appears to have been far more impressive than the three accommodations (*1*).

Was this reaction rational? It is surprisingly difficult to establish an advantage thesis: to show that predictions tend to provide stronger support than accommodations. The content of the hypothesis, of the statements needed to link the hypothesis to the observation, of background beliefs, and of the observation itself may all be unaffected by the question of whether the observation was accommodated or predicted, and these seem to be the only factors that can affect the degree to which a hypothesis is supported by evidence. The difference between accommodation and prediction seems merely one of timing. Some observations are more reliable and more telling than others, but the date when they were made seems to be irrelevant (*2*–*6*).

To make the case against the advantage thesis more vivid, consider a fictitious case of twin scientists. These twins independently and coincidentally generate the same hypothesis. The only difference between them is that one twin accommodates an observation that the other predicts. If there really were an advantage to prediction, we ought to say that the predictor has more reason to believe the hypothesis than the accommodator, though they share hypothesis, data, and background beliefs. This is counterintuitive, but things get worse. Suppose that the twins meet and discover their situation. It seems clear that they should leave the meeting with a common degree of confidence in the hypothesis they share. If they came to the meeting with different degrees of rational confidence in their hypothesis, at least one of them ought to leave with a revised view. But what level should they settle on: the higher one of the predictor, the lower one of the accommodator, or somewhere in between?

There seems no way to answer the question. Moreover, if there is a relevant difference between prediction and accommodation, then the twin who should revise her view after she meets her sibling must not revise simply because she knew all along that someone like her twin might have existed. If revision were in order merely because of this possibility, then the difference between accommodation and prediction would vanish. Whenever data are accommodated, we know that there might have been someone who produced the hypothesis earlier and predicted the data instead. But how can the question of whether there actually is such a person make any difference to our justified confidence in the hypothesis? Any adequate defense of the putative difference between prediction and accommodation will have to explain how an actual meeting could be different from a hypothetical meeting. Those who reject the distinction seem to be on firm ground when they maintain that no such explanation is possible.

Ad Hoc Hypotheses, Real Tests, and Best Explanations

Here are three popular defenses of the advantage thesis. One is that accommodation allows a hypothesis to be built around the data, but such a hypothesis would be ad hoc and hence only poorly supported. As it stands, this is not a good argument. The expression "ad hoc" literally means "purpose-built." To say that an accommodation is ad hoc in this sense is just to repeat that it is accommodation; it is not to say or to show that the hypothesis is poorly supported or otherwise deficient. On the other hand, the expression "ad hoc hypothesis" is often used in a derogatory sense that implies that the hypothesis is poorly supported. But on that reading, the argument becomes circular: Accommodating hypotheses are poorly supported, therefore they are poorly supported. On either reading, the ad hoc argument fails.

A second argument for the advantage thesis is the argument from testing. According to this argument, predictions are worth more than accommodations because it is only through its predictions that a hypothesis gets properly tested, and it is only by passing a test that a hypothesis gains genuine support. The idea is that a test is something that could be failed, and it is only a prediction that a hypothesis can fail. Here the hypothesis is made to commit in advance and say how things will be, so that we may go on to discover that things actually are not that way. So if the hypothesis passes the test of prediction, the hypothesis has earned some credit. This thought is closely related to Karl Popper's emphasis on the importance of getting hypotheses to stick their necks out, though Popper himself took the radical view that there is no such thing as positive evidence (*7*). In accommodation, by contrast, the hypothesis does not stick its neck out: It cannot be shown to be wrong, because the hypothesis is constructed after the data are known and compatibility is thus guaranteed.

An analogy helps to bring out the intuitive strength of the argument from testing (*8*). Suppose that Jacob, my elder son, takes his trusty bow and arrow and shoots at a target on the side of a barn, hitting the bull's-eye. We are duly impressed. Now Jonah, my younger son, steps up to a different barn, pulls back his bow, and shoots his arrow at the barn. Then he walks up to the side of his barn and paints a bull's-eye around his arrow. We would give him rather less credit, for archery anyway. Accommodation is like drawing the bull's-eye afterwards, whereas in prediction the target is there in advance. The argument from testing seems clearly to show why successful prediction should count more than accommodation.

Nevertheless, as it stands, this too is a poor argument. It confuses the scientific hypothesis with the scientist who formulates it. What is true is that only in the case of pre-

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diction does the scientist run the risk of having been mistaken, because it is only in the case of prediction that the scientist may have to admit to having made a false prediction. But we ought to concern ourselves here with the science, not with the scientist. When it comes to the hypothesis, there is no contrast between prediction and accommodation. If the data been different, the prediction would have been false and the hypothesis refuted or disconfirmed. But just the same goes for accommodated data: If they had been different, the hypothesis that was built around the actual data would also have been refuted. It is also true that, had the accommodated data been different, the scientist would have built a different hypothesis, but that is not to the point. As far as the specific hypothesis itself is concerned, the situation is symmetrical: If a prediction is a test, then so is an accommodation. So the argument from testing fails.

The third popular argument for the advantage thesis is the argument from the best explanation (*9*). In the case of accommodation, there are two explanations for the fit between hypothesis and data. One is that the hypothesis is true; another is that the hypothesis was designed to fit the data. We know that the second, the accommodation explanation, applies to the case; and this seems to preempt an inference to the truth explanation. In the case of prediction, by contrast, we know that the accommodation explanation does not apply, which leaves the truth explanation in the running. In one case, the truth explanation cannot be the best explanation, whereas in the other it might be. This is why prediction is better than accommodation.

This argument from the best explanation has considerable intuitive force but also a number of weaknesses. The most important of these is that it is unclear whether the accommodation explanation actually does preempt the truth explanation. They certainly could both be correct, and to assume that accepting the accommodation explanation makes it less likely that the hypothesis is true is once again to beg the question against accommodation. The issue is precisely whether the fact that a hypothesis was designed to fit the data in any way weakens the inference from the fit to the correctness of the hypothesis, and the argument from the best explanation does not help to settle it.

Choice and Fudging

Are there any better arguments for the advantage thesis? I will suggest two. To make sense of them, we need first of all to note that there are many relatively uncontroversial factors that affect the degree of support that an observation provides for a hypothesis (*10*, *11*). These can be roughly divided into features of the evidence and features of the hypothesis. On the list of evidential virtues, we may put down that more supporting evidence is better than less, but this is not the only entry. Variety in the data is also an evidential virtue. Someone who just repeats the same experiment over and over eventually reaches a point of diminishing returns, whereas a hypothesis supported by a variety of experiments rightly inspires greater confidence. Having accurate and precise data is another evidential virtue, as is having the results of controlled experiments, in which the scientist can be confident of the absence of disturbing influences. The same applies to so-called ''crucial'' experiments, in which the evidence simultaneously supports one hypothesis while undermining some of its rivals, and to evidence that would be very improbable unless the hypothesis were true.

One can also construct a list of theoretical virtues. One is the prior plausibility of the hypothesis: how natural it is and how well it fits with other claims that are already accepted. Simplicity is another theoretical virtue: With an appeal to Ockham's razor, the simpler hypothesis is often given a better chance of being correct. Other theoretical virtues include the plausibility of the auxiliary statements that have to be used to wring testable consequences out of the hypothesis, and the absence of good competing hypotheses.

The two arguments I want to make for the advantage thesis make connections between the contrast between prediction and accommodation and these relatively uncontroversial evidential and theoretical virtues. The first and simpler of the two arguments is the argument from choice. Scientists can often choose their predictions in a way in which they cannot choose which data to accommodate. When it comes to prediction, they can pick their shots, deciding which predictions of the hypothesis to check. Accommodated data, by contrast, are already there, and scientists have to make out of them what they can. But how can this be used to show that predictions tend to provide stronger support than accommodations? Whatever Karl Popper may have advised, scientists often try to make the strongest possible case for their own hypotheses. So they have a motive for choosing predictions which, if correct, will give maximum support to their hypotheses, not because they are predictions but because they will exhibit the sort of evidential virtues just mentioned. Thus, the scientist will choose predictions that allow for very precise observations that would substantially increase the variety of data supporting the hypothesis, and so on. Successful predictions tend to provide stronger support than accommodations, not directly because they are predictions, but indirectly because scientists have control over which predictions to check; control that is not available in the case of accommodation. The earlier availability of the hypothesis may lead to the gathering of different data and to data that yield stronger support.

That is the argument from choice. It is relatively straightforward, but it does not show quite as much as one might hope. For one thing, scientists do not always have that much choice about what predictions to check: Halley is a case in point. Nevertheless, the argument from choice does show why successful predictions tend sometimes to be more powerful than accommodations, and more powerful because they are predictions. This is what we might call the weak advantage thesis. The point is that the scientist can sometimes use a hypothesis as a guide to data that would provide particularly strong support in the case of prediction, but not in the case of accommodation, because the hypothesis is only antecedently available in the case of prediction. Unfortunately, the argument from choice does not give a reason for the more ambitious claim—the strong advantage thesis—that a single, particular observation that was accommodated would have provided more support for the hypothesis in question if it had been predicted instead. The following analogy may help to clarify this distinction between the weak and strong advantage theses. The fact that I can choose what I eat in a restaurant but not when I am invited to someone's home explains why I tend to prefer the dishes I eat in restaurants over those I eat in other people's homes, but this obviously gives no reason to suppose that lasagna, say, tastes better in restaurants than in homes. Similarly, the argument from choice may show that predictions tend to provide stronger support than accommodations, but it does not show that the fact that a particular datum was predicted gives any more reason to believe the hypothesis than if that same datum had been accommodated. To defend this strong advantage thesis, we need another argument: the ''fudging'' argument.

The fudging argument depends on an interesting feature of the two lists of virtues given above, namely that some of the evidential virtues are in tension with some of the theoretical virtues. Here is an example. On the evidence side, scientists want the supporting evidence to be extensive and varied. On the theoretical side, they want the simplest hypothesis. It is easy to have either one of these virtues on its own. If one just wants lots of varied evidence, one can assemble an encyclopedia of facts; but the hypothesis that is their conjunction will be incredibly complex because the facts are so heterogeneous. On the other hand, if all that matters is simplicity, that too is easy, so long as one does not mind about covering much evidence. What is hard and what scientists want is to satisfy both constraints simultaneously. They want simple hypotheses that nevertheless handle a great diversity of evidence.

Now for the fudging argument. When scientists have data to accommodate, they do the best they can. If the data are diverse, however, this can lead to a sacrifice in simplicity and other theoretical virtues. The epicycles that Ptolemaic astronomers were forced to insert into their planetary model in order to account for the available astronomical data are often taken to be a blatant case of fudging (*12*).

Practicing scientists ought to be able to generate more subtle examples from the recent histories of their own disciplines. The point is that the investigator may, sometimes without fully realizing it, fudge the hypothesis, putting in a few extra epicycles or kinks to ensure that more of the data gets captured. In a case of prediction, by contrast, there is no motive to introduce anything unnatural into the hypothesis, because the investigator does not know the right answer in advance and so would not know what kink to introduce even if one were required. So in that case, the scientist will use the best hypothesis and, if the prediction is successful, will have manifested both empirical and theoretical virtues.

The advantage that the fudging argument attributes to prediction is thus in some respects similar to the advantage of a double-blind medical experiment, in which neither the doctor nor the patients know which patients are getting the placebo and which are getting the drug being tested. The doctor's ignorance makes her judgment more reliable, because she does not know what the "right" answer is supposed to be. The fudging argument makes an analogous suggestion about scientists generally. Not knowing the right answer in advance—the situation in prediction but not in accommodation—makes it less likely that the scientist will fudge the hypothesis in a way that makes for poor empirical support (*13*, *14*).

The claim that the need to accommodate data may lead to fudging ought to be uncontroversial, but does it really show that scientists ought to give any weight to whether the data were accommodated or predicted? A counterargument is that whatever fudging may occur, this is something the scientists can determine directly, by inspection of the hypothesis and the data, once these are given. But this may be to exaggerate scientists' abilities or equivalently to underestimate the complexity of the factors that determine the degree to which the hypothesis is supported by data. Fudging may be neither fully conscious nor readily visible, so the indirect evidence that information about whether the evidence was accommodated or predicted provides may be relevant.

The Twins Revisited

From the perspective of the fudging argument, we are now in a position to see the germs of truth in the three popular arguments for the advantage thesis that we considered above. Recall first the argument that accommodations are worse than predictions because accommodations are ad hoc. My objection was that this leaves the question unanswered or begged, because calling a hypothesis ad hoc either just means it is designed to accommodate, or it simply asserts that an accommodating hypothesis cannot enjoy strong empirical support. The fudging argument provides an independent reason why accommodating systems tend to be ad hoc in the second sense (only weakly supported by the evidence they accommodate); namely, that they tend to suffer from theoretical vices such as excessive complexity and poorly motivated assumptions.

The second argument that I initially rejected was the testing argument: that predictions are better because only they test the hypothesis, because a test is something that can be failed. My objection was that although it may be that only predictions test the scientist, an accommodating hypothesis can be as falsifiable as a predicting one. In such a case, if the accommodated evidence had been different, the hypothesis would have been disconfirmed. The fudging argument is related to the notion that we should after all test the scientist and that she should test herself. It is not simply that she ought to run the risk of being wrong, though that is a consequence of the real point: She should place herself in a situation where she does not know the right answer in advance, because she is then less likely to fudge.

Lastly, the fudging argument suggests an improved version of the argument from the best explanation. In its original form, that argument has it that prediction is better because the best explanation for predictive success is truth, whereas the best explanation for accommodation is instead that the hypothesis was designed for that purpose. My objection was that it was not clear that the accommodating explanation preempts the truth explanation. It is true that only in cases of accommodation can we explain the fit between hypothesis and observation by pointing out that the hypothesis was designed for the purpose, but whether this makes it less likely that the hypothesis is correct is just what we want to know. In contrast, it is clear that the fudging argument competes with the truth explanation. Insofar as we may reasonably infer the explanation that the fit between hypothesis and observation in the case of accommodation is due to fudging, this undermines our confidence in the inference from fit to truth. So the best-explanation account of the difference between accommodation and prediction can be salvaged by replacing the accommodation explanation with the fudging explanation. In cases of accommodation, the inference from fit to truth may be blocked by the inference from fit to the conclusion that the theoretical system is ad hoc, in the pejorative sense.

The fudging argument also gives us an answer to the puzzle of the twin scientists with which this article began and which seemed to show that there could be no relevant difference between prediction and accommodation. We have two scientists who happen to come up with the same hypothesis; where one accommodates evidence, the other predicts. After they compare notes, they should have a common level of confidence in the hypothesis they share. The difficulty was that it seemed impossible to say what level this should be, and how meeting a predictor could be any different for the accommodator than knowing what all accommodators know: that if someone had produced the same hypothesis with less evidence, the prediction of the balance of the data would have been successful.

The answer to this puzzle is now clear. The accommodator ought to worry that she had to do some fudging, but her suspicion is defeasible. One of the things that can defeat it, though not common in the history of science, is meeting a corresponding predictor. If the accommodator meets someone who predicted data she only accommodated, with the same independently produced hypothesis, this shows that the accommodator probably did not fudge to make those accommodations. The predictor, ignorant of the data he predicted, had no motive to fudge the hypothesis to get those results; consequently, the fact that the predictor came up with just the same hypothesis provides independent evidence that the accommodator did not fudge either. It is more likely that neither the predictor nor the accommodator fudged than that they both did. At the same time, merely knowing that the same hypothesis could have been constructed earlier is not relevant, because such a construction might have then required arbitrary and unmotivated fudging. A predictor might have come up instead with a different and better hypothesis. If an actual meeting takes place, however, the twins should leave it sharing the higher confidence of the predictor. As for those of us without scientific siblings, what the fudging argument shows is that we are sometimes justified in being more impressed by predictions than by accommodations.

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The First Glacial Maximum in North America

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The regular advance and retreat of continental ice sheets is a defining feature of the past several million years of Earth history. Despite widespread glacial sediments in Europe, Asia, and North America, however, most of what we know about the timing and extent of ice sheets before the most recent ones comes from marine oxygen-isotope ($\delta^{18}O$) records. This is because there are few methods for dating terrestrial glacial deposits that are too old for ^{14}C or luminescence dating techniques. Marine isotope records reflect global ice volume and rarely identify the location of the ice, so it is seldom possible to associate individual terrestrial glacial deposits with particular marine isotope stages. This presents a challenge to understanding long-term ice sheet and climate dynamics. Here we use an example from North America to describe a way to overcome this difficulty, by using the cosmic ray–produced radionuclides ^{26}Al and ^{10}Be to date sequences of intercalated paleosols and tills.

These two nuclides are produced at a fixed ratio in quartz that is exposed to cosmic rays, but they decay at different rates. If sedimentary quartz is exposed long enough that nuclide concentrations reach a balance between production and loss by decay and surface erosion and is then buried and removed from the cosmic ray flux, ^{26}Al and ^{10}Be measurements can yield the duration of burial and the erosion rate before burial. This technique has been used to date river sediment buried in caves (*1*); we adapted it to more complicated stratigraphic situations (*2*). It is most accurate when sediments are exposed for long periods of time (>10^5 years) and then buried rapidly to at least several meters' depth. This situation arises when soils develop during long periods of landscape stability and are then buried by till during ice sheet advances. In this case, the ^{26}Al and ^{10}Be concentrations in the buried soil tell us the age of the overlying till.

At the Musgrove clay pit in central Missouri (Fig. 1), two tills, the Atlanta and Moberly formations, overlie deeply weathered shale and limestone as well as locally derived colluvium of the Whippoorwill formation. Each till is capped by a paleosol; thus, the section records a long period of weathering and slow erosion before glaciation, followed by at least two ice sheet advances with an intervening period of soil formation (fig. S1) (*3*). We measured ^{26}Al and ^{10}Be in quartz from paleosols in the Whippoorwill and Atlanta formations (table S1) and found that the Atlanta till was deposited 2.41 ± 0.14 million years ago (Ma). The Whippoorwill paleosol has unusually high nuclide concentrations, which allows accurate measurements and ensures that any slow production of nuclides after burial is insignificant compared to the large nuclide inventory produced before burial. This minimizes the effect of uncertainties in the burial history and in the nuclide production rates on our inferred age for the Atlanta till. Samples from the Atlanta paleosol had lower nuclide concentrations, leading to larger uncertainties, and determining the age of the Moberly till from these samples required assumptions about the initial nuclide concentration in the Atlanta till. Given a conservative range of concentrations, the Moberly till is 1.6 to 1.8 million years old.

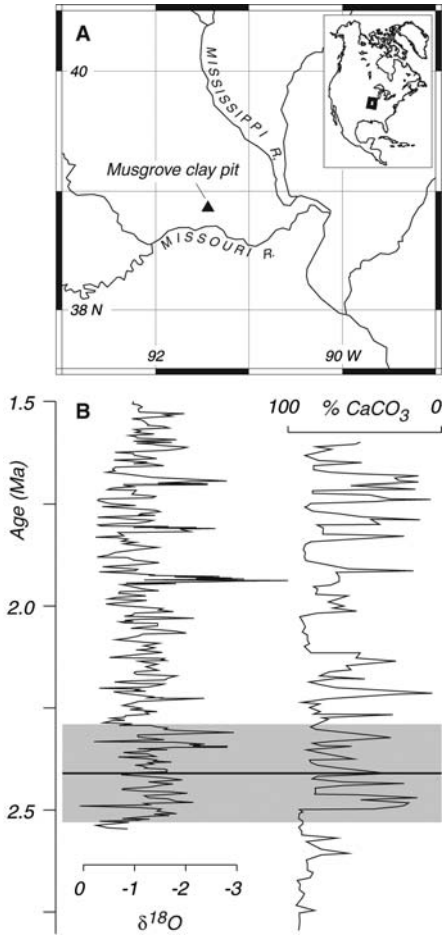

Fig. 1. (A) The location of Musgrove pit. (B) Marine records suggesting early advances of the Laurentide Ice Sheet: the $\delta^{18}O$ record from Ocean Drilling Program site 625A in the Gulf of Mexico (*4*) and the carbonate percentage at Deep-Sea Drilling Program site 552A in the North Atlantic, which is inversely related to the concentration of ice-rafted debris (*5*). The horizontal line and the shaded band denote our age estimate for the Atlanta till and ±1σ uncertainty. We have adjusted the time scales of the $\delta^{18}O$ (*4*) and $CaCO_3$ (*5*) records to account for revisions to the magnetic polarity time scale (*2*); however, the two time scales may differ in this age range.

The Atlanta till is thus the oldest direct evidence of continental glaciation in the Northern Hemisphere. It records the first, and nearly the most southerly, advance of the Laurentide Ice Sheet. Given our dating uncertainty, this is likely the same advance suggested by negative $\delta^{18}O$ excursions in Gulf of Mexico sediments of similar age (*4*), although the present uncertainties in the half-lives of ^{26}Al and ^{10}Be (*2*), as well as in the time scales for the marine records, make it difficult to correlate either event with the major increase in North Atlantic ice-rafted debris near 2.5 Ma (shown in Fig. 1 by the abrupt decrease in $CaCO_3$ percentage) (*5*). The idea that Northern Hemisphere continental ice sheets first formed 2.7 to 2.4 Ma has previously been based on inference from marine sediments. The Atlanta till is direct terrestrial evidence that the Laurentide Ice Sheet did in fact develop and advance to its full extent during this time interval.

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Materials and Methods
Fig. S1
Table S1
References and Notes

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A Diarylquinoline Drug Active on the ATP Synthase of *Mycobacterium tuberculosis*

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The incidence of tuberculosis has been increasing substantially on a worldwide basis over the past decade, but no tuberculosis-specific drugs have been discovered in 40 years. We identified a diarylquinoline, R207910, that potently inhibits both drug-sensitive and drug-resistant *Mycobacterium tuberculosis* in vitro (minimum inhibitory concentration 0.06 μg/ml). In mice, R207910 exceeded the bactericidal activities of isoniazid and rifampin by at least 1 log unit. Substitution of drugs included in the World Health Organization's first-line tuberculosis treatment regimen (rifampin, isoniazid, and pyrazinamide) with R207910 accelerated bactericidal activity, leading to complete culture conversion after 2 months of treatment in some combinations. A single dose of R207910 inhibited mycobacterial growth for 1 week. Plasma levels associated with efficacy in mice were well tolerated in healthy human volunteers. Mutants selected in vitro suggest that the drug targets the proton pump of adenosine triphosphate (ATP) synthase.

After AIDS, tuberculosis (TB) is the leading cause of infectious disease mortality in the world, with 2 million to 3 million deaths per year (*1*). The TB and HIV epidemics fuel one another in coinfected people, and at least 11 million adults are infected with both pathogens (*2*, *3*). Hence, factors contributing to the TB burden include not only difficulties in implementing TB control programs in many countries but also the recent increase in the number of HIV-infected individuals (*4*). Although current first-line anti-TB drug regimens can achieve more than 99% efficacy, this is often reduced because of drug resistance (*5*). As pointed out by the Global Alliance for TB Drug Development, new drugs that could shorten or simplify effective treatment of TB would substantially improve TB control programs (*6*).

We report on the antimycobacterial properties of the diarylquinolines (DARQs). The lead compound, R207910, not only has several properties, both in vitro and in vivo, that may improve the treatment of TB, but also appears to act at a new target, providing an antimycobacterial spectrum different from those of current drugs. Its clinical potential is being tested in patients.

Chemistry and in vitro antimycobacterial activity. We sought new anti-TB compounds by selecting prototypes of different chemical series and testing them for inhibition of multiple-cycle growth of *Mycobacterium smegmatis*. A whole-cell assay was preferred because of its ability to concurrently assess multiple targets. Chemical optimization of a lead compound led to a series of DARQs with potent in vitro activity against several mycobacteria, including *M. tuberculosis* (*7*). To date, 20 molecules of the DARQ series have a minimum inhibitory concentration (MIC) below 0.5 μg/ml against *M. tuberculosis* H37Rv. Antimycobacterial activity was confirmed in vivo for three of these compounds (*8*). The most active compound of the class, R207910, is a pure enantiomer with two chiral centers. A mixture of diastereoisomers is prepared in five steps, and R207910 is isolated from the resulting mixture of four isomers; Fig. 1 shows its structure and absolute configuration. Its chemical name is 1-(6-bromo-2-methoxy-quinolin-3-yl)-4-dimethylamino-2-naphthalen-1-yl-1-phenyl-butan-2-ol; the molecular formula is $C_{32}H_{31}BrN_2O_2$ and the molecular weight is 555.51 daltons.

Structurally and mechanistically, DARQs are different from both fluoroquinolones (including methoxyquinolones) and other quinoline classes, including mefloquine and its analogs 4-methylquinolines and 4-quinolylhydrazones (*9*–*13*). One of the major structural differences between DARQs and other quinolone or quinoline classes is the specificity of the functionalized lateral (3′) chain borne by the DARQ class.

R207910 has a unique spectrum of potent and selective antimycobacterial activity in vitro (Table 1). The range of MICs for the international reference strain *M. tuberculosis* H37Rv and six fully antibiotic susceptible isolates was 0.030 to 0.120 μg/ml (Table 1), versus 0.500 μg/ml for rifampin and 0.120 μg/ml for isoniazid (*14*). R207910 demonstrated similar in vitro efficacy against *M. tuberculosis* clinical isolates resistant to the TB agents isoniazid, rifampin, streptomycin, ethambutol, pyrazinamide, and moxifloxacin. R207910 did not inhibit *M. tuberculosis* purified DNA gyrase, the target for quinolones.

The lack of cross-resistance with currently used anti-TB agents suggests that R207910 retains activity against multidrug-resistant (MDR) TB strains. Indeed, using the BACTEC culture system (*15*), we observed inhibition of bacterial growth when

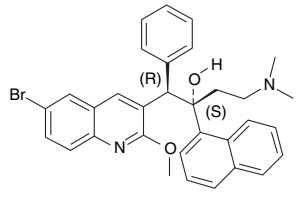

Fig. 1. Absolute configuration of R207910. From the racemate, two diastereoisomers were purified and separated with a ratio A:B = 40:60 by high-performance liquid chromatography (HPLC). The active diastereoisomer (A) was separated into the corresponding (*R*,*S*) (A1) and (*S*,*R*) (A2) isomers by chiral HPLC. The active (*R*,*S*) isomer is R207910 (A1). The structure of R207910 in the solid state was solved by circular dichroism and x-ray crystallography experiments. The absolute configuration of two asymmetric centers was determined as *R*,*S* (the carbon bearing the phenyl substituent is *R* and the carbon bearing the hydroxyl substituent is *S*).

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MDR-TB strains were exposed to fixed concentrations of R207910. All 30 isolates of MDR-TB tested were susceptible to R207910 at 0.100 μg/ml; of these, 17 (57%) were susceptible to R207910 at 0.010 μg/ml (Table 2). Using the same method, we observed similar susceptibility among 10 fully antibiotic-susceptible strains. Low MICs were also found for other mycobacterial species, including *M. bovis*, *M. kansasii*, and *M. ulcerans*, as well as species naturally resistant to many other anti-TB agents and involved in opportunistic infections, such as *M. avium* complex (MAC), *M. abscessus*, *M. fortuitum*, and *M. marinum* (Table 1).

The activity of R207910 appeared to be specific for mycobacteria. R207910 had much higher MICs for *Corynebacterium* and *Helicobacter pylori* (MIC 4.0 μg/ml), and especially for other organisms such as Gram-positive *Nocardia*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis*, or Gram-negative *Escherichia coli* and *Haemophilus influenzae* (table S1).

Exposure of *M. tuberculosis* in log-phase growth to concentrations of R207910 at 10 times MIC resulted in a reduction in bacterial load of 3 log units after 12 days, indicating that R207910 has bactericidal activity in vitro (table S2). The killing effect was not increased by higher concentrations of the compound, which suggests that the killing was time-dependent rather than concentration-dependent.

Isolation of mutants, cross-resistance, and postulated drug target. Mutants of *M. tuberculosis* and *M. smegmatis* resistant to R207910 were selected in vitro to quantify the proportion of resistant mutants arising (by comparison with rifampin), to assess the cross-resistance pattern, and to investigate the mechanism of action.

From selection experiments, the proportion of resistant mutants that emerged was 5×10^{-7} and 2×10^{-8} at 4 times MIC, and 5×10^{-8} and 1×10^{-8} at 8 times MIC, for *M. tuberculosis* and *M. smegmatis*, respectively (*8*). In the case of *M. tuberculosis*, these proportions were comparable to those of rifampin-resistant mutants that emerged (10^{-7} to 10^{-8}). In addition, the susceptibility of R207910-resistant *M. tuberculosis* strains remained unchanged relative to other anti-TB agents, including isoniazid, rifampin, streptomycin, amikacin, ethambutol, and moxifloxacin. Analysis of R207910 mutants of *M. tuberculosis* and *M. smegmatis* showed that there were no mutations in the DNA gyrase regions *gyrA* and *gyrB*, sequences in which quinolone resistance typically develops.

The genomes of the resistant *M. tuberculosis* strain BK12 and the two resistant *M. smegmatis* strains R09 and R10, as well as the parental *M. smegmatis*, were sequenced to near-completion. Point mutations that conferred R207910 resistance were identified by comparative analysis of the genome sequences of susceptible and resistant strains of *M. tuberculosis* and *M. smegmatis* (Fig. 2). The only gene commonly affected in all three independent mutants encodes atpE, a part of the F0 subunit of ATP synthase. This finding indicates that R207910 inhibits the proton pump of *M. tuberculosis* ATP synthase. The two point mutations identified—$Asp^{32} \rightarrow$ Val (D32V) for *M. smegmatis* and $Ala^{63} \rightarrow$ Pro (A63P) for *M. tuberculosis*—are both in the membrane-spanning domain region of the protein.

Complementation studies have verified that the mutant *atpE* gene is responsible for resistance to R207910, implying that the *atpE* gene product is the target of R207910 in mycobacteria. Wild-type *M. smegmatis* was transformed with a construct expressing the F0 subunit from mutant *M. smegmatis* R09 (D32V). This rendered the cells resistant to R207910, with a MIC identical to that of the R207910-resistant strain *M. smegmatis* R09 (D32V) (table S3). In addition, when the plasmid was reisolated from these transformants and the ATP F0 operon was sequenced, it was shown to have retained the mutant allele (D32V).

Pharmacokinetic studies in mice. After single or multiple oral administration in male Swiss SPF mice, R207910 was rapidly absorbed. After a single dose of 6.25 mg/kg body weight, maximum plasma concentrations (C_{max}) reached 0.40 to 0.54 μg/ml within 1 hour; after a dose of 25 mg/kg, C_{max} reached 1.1 to 1.3 μg/ml within 2 to 4 hours. Areas under the curve for concentration versus time (AUCs) were 5.0 to 5.9 μg·hour/ml after a dose of 6.25 mg/kg; AUCs were 18.5 to 19.4 μg·hour/ml after a dose of 25 mg/kg

Table 1. MICs of R207910 that inhibited 99% of the growth of different mycobacterial species.

Mycobacterial species	Number of strains	Range of MICs for multiple strains (μg/ml)	Median MIC (μg/ml)
M. tuberculosis, H37Rv	1	—	0.030
M. tuberculosis, fully susceptible clinical isolates	6	0.030–0.120	0.060
M. tuberculosis resistant to isoniazid	7	0.003–0.060	0.010
M. tuberculosis resistant to rifampin	1	—	0.030
M. tuberculosis resistant to isoniazid and rifampin	2	0.030–0.030	0.030
M. tuberculosis resistant to isoniazid and streptomycin	1	—	0.010
M. tuberculosis resistant to ethambutol	1	—	0.010
M. tuberculosis resistant to pyrazinamide	1	—	0.030
M. tuberculosis resistant to fluoroquinolone	2	0.060–0.120	0.090
M. bovis	1	—	0.003
M. avium/M. intracellulare (MAC)	7	0.007–0.010	0.010
M. kansasii	1	—	0.003
M. marinum	1	—	0.003
M. fortuitum	5	0.007–0.010	0.010
M. abscessus	1	—	0.250
M. smegmatis	7	0.003–0.010	0.007
M. ulcerans	1	—	0.500

Table 2. Susceptibility of drug-resistant *M. tuberculosis* to two concentrations of R207910, as measured by the BACTEC radiometric system.

Resistance pattern	Total number of strains	Number of strains inhibited by 0.100 μg/ml	Number of strains inhibited by 0.010 μg/ml
M. tuberculosis, fully susceptible isolates	10	10	1 (10%)
M. tuberculosis, resistant isolates	40	40	22 (55%)
Multidrug-resistant *M. tuberculosis* (resistant to at least rifampin and isoniazid)	30	30	17 (57%)
M. tuberculosis resistant to isoniazid, rifampin, streptomycin, and ethambutol	13	13	8 (62%)
M. tuberculosis resistant to isoniazid	38	38	20 (53%)
M. tuberculosis resistant to rifampin	30	30	17 (57%)
M. tuberculosis resistant to streptomycin	25	25	15 (60%)
M. tuberculosis resistant to ethambutol	20	20	12 (60%)

(table S4). R207910 was extensively distributed to tissues, including lungs and spleen (Fig. 3A) (table S4). Half-lives ranged from 43.7 to 64 hours in plasma and 28.1 to 92 hours in tissues (table S4). No accumulation of R207910 was observed after five daily oral doses, indicating that slow redistribution from tissues contributed to the relatively long half-lives in plasma. The long half-lives and consequent prolonged exposure are important factors determining the duration of activity in vivo and provide a rationale for less frequent dosing regimens.

To better understand the dose-response and exposure-response relationships between R207910 and TB infection, we combined data from in vivo efficacy and separate pharmacokinetic studies in mice. A single-dose pharmacokinetic study comparing C_{max} values and AUCs after doses of 6.25, 25, and 100 mg/kg confirmed that AUCs showed a better correlation with dose than did C_{max} values, reflecting limitations in the rate of absorption at higher dosing regimens. Dose linearity was better for tissues, in terms of both C_{max} and AUC. The data suggest that maintaining average plasma levels of 0.3 μg/ml throughout a dosing interval of 24 hours—which is achieved with a dose of 100 mg/kg per week—is necessary to achieve the optimal effect of monotherapy in mice infected with strain H37Rv.

Pharmacodynamic studies in mice. A single dose of R207910 (50 mg/kg) in the nonestablished infection murine model of TB infection resulted in a bacteriostatic effect [decrease of 0.5 log units in bacterial load in the lungs, in terms of colony-forming units (CFU)]; this effect lasted for 8 days and was similar to the effect of rifapentine (10 mg/kg) (Fig. 3B). By contrast, at 100 mg/kg, a single dose of R207910 had a bactericidal effect (decrease of up to 2.5 log units in bacterial load in the lungs) that lasted for 8 days, after which bacterial growth resumed at a rate similar to that seen in the controls. The extended effect of a single dose provides further support for a dosing regimen less frequent than five times per week.

In mouse studies using oral treatment of R207910 for 5 days per week for 4 weeks, starting the day after inoculation, the minimal dose of R207910 that prevented mortality in nonestablished infection was 1.5 mg/kg, and the minimal effective dose (MED) preventing gross lung lesions was 6.5 mg/kg. In mice receiving doses of 12.5 mg/kg, the bacterial load per organ was reduced from 5 to 2 log units ($P < 0.0014$) (Fig. 4A). Thus, the minimal bactericidal dose was very close to the MED; this finding confirms the time-

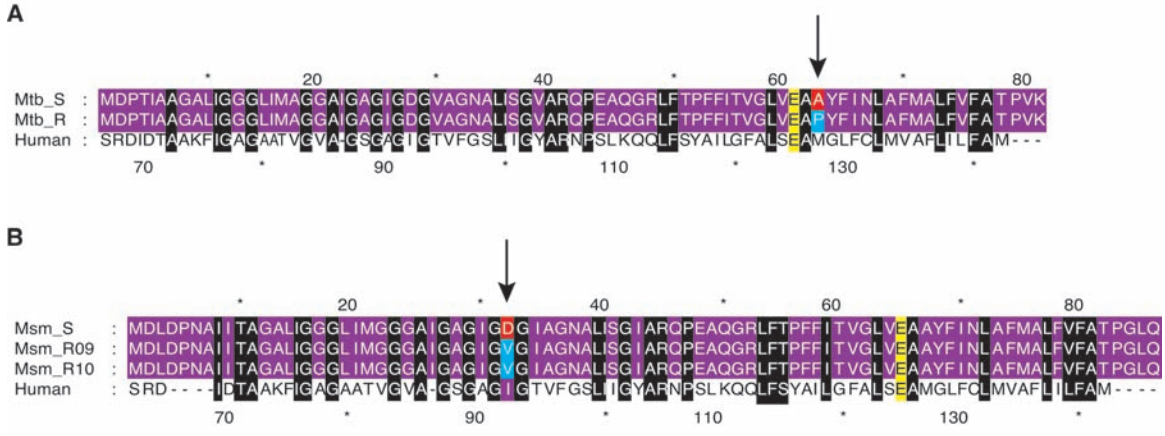


Fig. 2. atpE protein sequence alignments for *M. tuberculosis* and *M. smegmatis* mutants. (**A**) Mtb_S: drug-sensitive strain of *M. tuberculosis* H37Rv, atpE (residues 1 to 81; Swiss-Prot accession number Q10598). Mtb_R: drug-resistant strain of *M. tuberculosis* BK12, atpE (1 to 81; EMBL accession number AJ865377). *Homo sapiens* ATP5G3 (66 to 142; accession number Ensembl ENSP00000284727). (**B**) Msm_S: drug-sensitive strain of *M. smegmatis*, atpE (1 to 86; EMBL accession number AJ862722). Msm_R09 and Msm_R10: drug-resistant strains of *M. smegmatis* atpE (1 to 86; EMBL accession numbers AJ865378 and AJ865379, respectively). *Homo sapiens*, ATP5G3 (66 to 142; accession number Ensembl ENSP00000284727). Shading indicates amino acid similarity using BLOSUM62 matrix (*19*) (black, high; purple, medium). Yellow highlight indicates the proton-binding glutamic acid; red highlight indicates the amino acid of the sensitive strain (arrow); blue highlight indicates the amino acid of the resistant strain (arrow). Single-letter abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

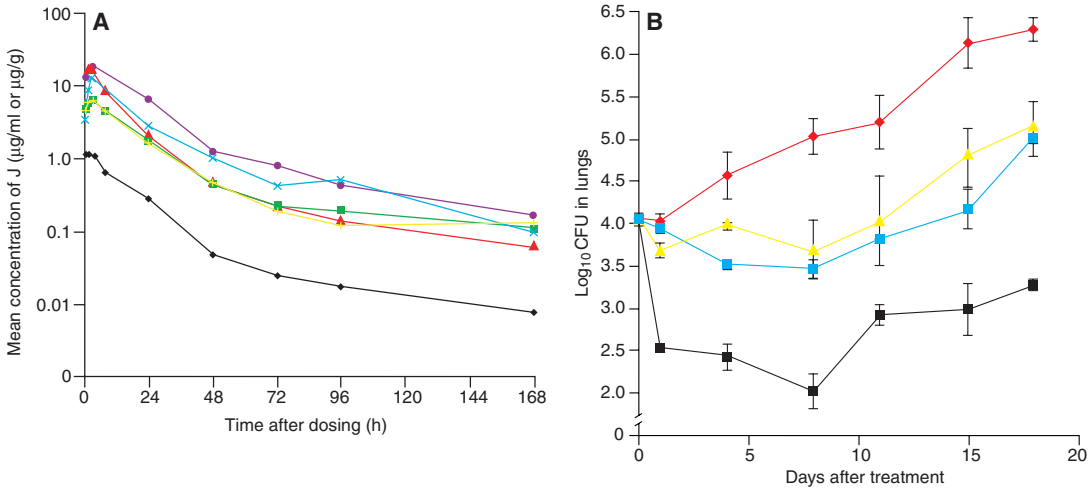


Fig. 3. (**A**) Mean plasma and tissue concentrations of R207910 (J) in mice ($n = 2$) after a single oral dose of J at 25 mg/kg. Black diamonds, plasma; red triangles, liver; blue crosses, kidney; green squares, heart; yellow crosses, spleen; purple circles, lung. (**B**) Effect of a single oral dose of J in the nonestablished infection murine TB model. Red diamonds, controls (untreated mice); blue squares, rifapentine (10 mg/kg); yellow triangles, J (50 mg/kg); black squares, J (100 mg/kg). Values are means ± SD.

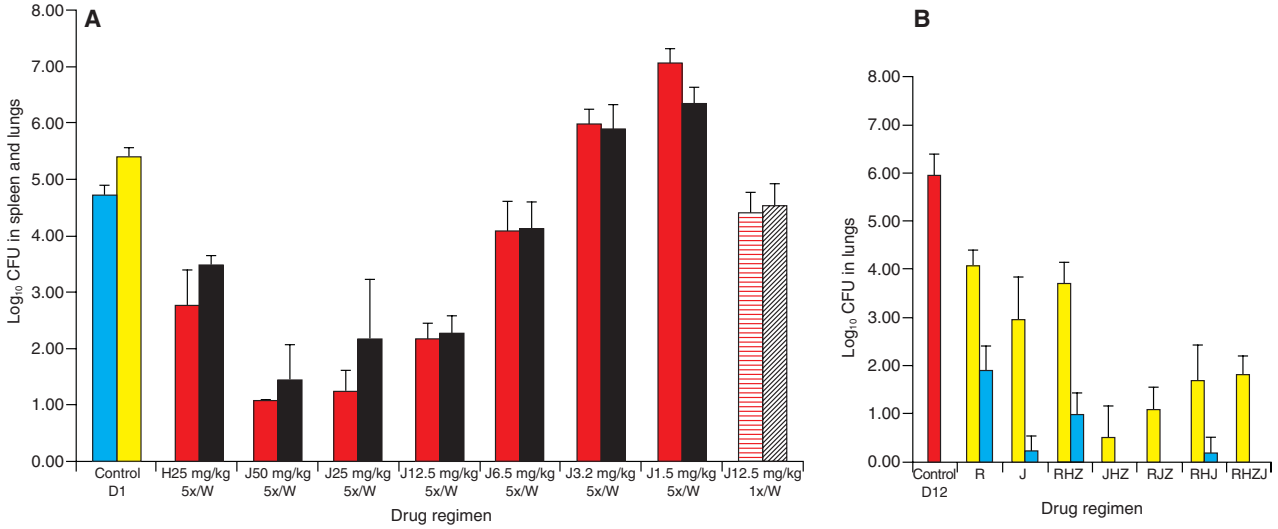


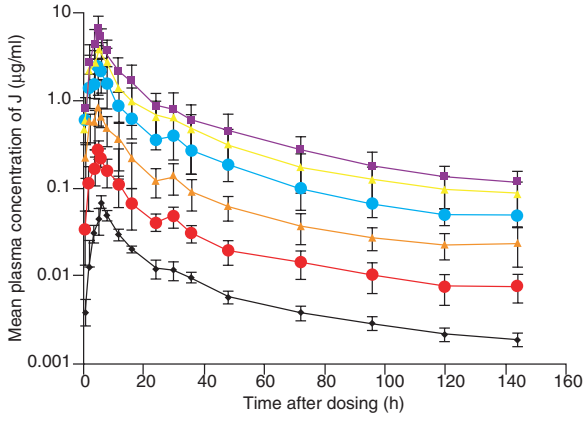
Fig. 4. (**A**) Minimal effective oral dose of R207910 (J) and effect of dosing frequency in the nonestablished infection murine TB model. Data shown were obtained from mice killed on day 28. Treatment began on day 1 and was administered 5 times per week (5×/W) for 4 weeks, except for one group treated with J (12.5 mg/kg) once per week (1×/W; hatched bars). Blue bar, control lung; yellow bar, control spleen (both at day 1); red bars, treatment lung; black bars, treatment spleen; H, isoniazid (25 mg/kg). Values are means ± SD. Multiple comparisons among pairs of groups were performed by Bonferroni's method (*20*). There were significant differences ($P < 0.0014$) between the control and J 12.5, J 25, or J 50 mg/kg (5×/W); between H and J 12.5, J 25, or J 50 mg/kg (5×/W); between J 12.5 mg/kg and J 25 or J 50 mg/kg (5×/W); and between J 12.5 mg/kg (1×/W) and J 12.5 mg/kg (5×/W). (**B**) Efficacy of R207910 (J) in the established infection murine TB model. Red bar, mice killed on day 12 just before treatment started; yellow bars, bacterial load in lungs of mice killed on day 42 (after 1 month of therapy); blue bars, bacterial load in lungs of mice killed on day 70 (after 2 months of therapy). Drugs were administered 5 times per week: R, rifampin (10 mg/kg); J, R207910 (25 mg/kg); H, isoniazid (25 mg/kg); Z, pyrazinamide (150 mg/kg). Values are means ± SD. Multiple comparisons among pairs of groups were performed by Bonferroni's method (*20*). There were significant differences ($P < 0.0018$) between the control and all treatment groups after 1 and 2 months, between the RHZ combination and any combination containing J after 1 month, and between the RHZ combination and the RJZ and RHZJ combination after 2 months.

dependent rather than concentration-dependent activity of R207910 in vitro. At 25 mg/kg, the activity of R207910 was significantly better than at 12.5 mg/kg ($P < 0.0014$).

At 12.5 and 25 mg/kg, R207910 was significantly more active ($P < 0.0014$) than isoniazid (25 mg/kg), a drug known for its strong, early bactericidal activity (Fig. 4A). Moreover, at 12.5 mg/kg, a once-weekly dose of R207910 was almost as efficacious as a dose of 6.5 mg/kg given five times per week (Fig. 4A). This is likely a consequence of the long half-life of R207910.

In established infection (treatment beginning 12 to 14 days after inoculation when bacterial load was 5.94 log units), R207910 (25 mg/kg) as monotherapy was at least as active as the triple combination therapy RHZ [rifampin (R) + isoniazid (H) + pyrazinamide (Z)] and more active than rifampin alone (Fig. 4B). However, concern about resistance development would preclude clinical use of R207910 as monotherapy. When added to the first-line triple TB therapy combination RHZ, R207910 (25 mg/kg) yielded a greater decrease in bacterial load in the lungs (relative to the standard RHZ treatment regimen) by 2 log units after 1 month of therapy and by a further 1 log unit after 2 months of therapy ($P < 0.0018$ in both cases). When substituting each first-line drug of the RHZ combination with R207910 (25 mg/kg), the activity of each combination containing R207910 (J) was significantly improved relative to that of RHZ, particularly after 1 month of treatment ($P < 0.0018$). In addition, after 2 months of treatment with JHZ and RJZ, the lungs of all animals were culture-negative. The differences among the bactericidal activities of the combinations RHJ, JHZ, and RJZ were not significant. The bactericidal activity obtained by the RHZ combination after 2 months of therapy was matched by the combinations JHZ and RJZ after just 1 month of therapy.

Fig. 5. Mean profiles of plasma concentration versus time for R207910 (J) after single oral administration in healthy male subjects immediately after a meal. Dose of J: black diamonds, 10 mg; red circles, 30 mg; orange triangles, 100 mg; blue circles, 300 mg; yellow triangles, 450 mg; purple squares, 700 mg (n = 6 per dose studied). Values are means ± SD.



Pharmacokinetic studies in humans. Preclinical safety assessment (including 28-day toxicology in rats and dogs, genetic toxicology, and safety pharmacology) supported the administration of R207910 to humans. In the first clinical study, we explored the pharmacokinetics, safety, and tolerability of R207910 in healthy male subjects in a double-blind, randomized, placebo-controlled design (*8*). The mean plasma concentration–time profiles after single-dose oral administration of R207910 (escalating doses, 10 to 700 mg R207910) are shown in Fig. 5.

Pharmacokinetic results from the single-ascending-dose study suggest that R207910 was well absorbed after a single oral administration and that the peak concentration was reached at 5 hours (median value) after the dose. After C_{max} was reached, the drug concentration declined triexponentially with time. The pharmacokinetics of R207910 were linear up to the highest dose tested

(700 mg); both C_{max} and AUC increased proportionally with the administered dose. There was no dose-dependent change in the terminal half-life.

Data from a multiple-ascending-dose study (once-daily doses of R207910 at 50, 150, and 400 mg/day) have shown an increase by a factor of ~2 in the AUC from the dose time to 24 hours later (AUC_{0-24h}) between day 1 and day 14. There was no substantial variation between subjects. This suggests an ''effective half-life'' of 24 hours. The mean AUC_{0-24h} values were 7.91, 24, and 52 μg·hour/ml at steady state (corresponding to average concentrations of 0.33, 1.0, and 2.2 μg/mL across a dosing interval) with 50, 150, and 400 mg/day, respectively. These average concentrations were greater than the concentration that achieved optimal activity in established infection in mice.

Safety and tolerability in humans. In the first single-dose clinical study, the only adverse events were mild or moderate in severity and were experienced by subjects receiving R207910 and placebo (*8*). The majority of adverse events reported (*8*) were considered possibly related to the study medication: placebo (seven of 18 subjects) or R207910 (20 of 36 subjects). In the second study, the good tolerability was maintained; only one subject withdrew from the study, because of a urinary tract infection (unrelated to R207910) after seven doses at 150 mg/day. There were no consistent trends in the vital signs, electrocardiograms, or laboratory safety tests in any of the cohorts.

Discussion. The DARQ R207910 is a member of a new chemical class of antimycobacterial agents and has a MIC equal to or lower than that of reference compounds. Its spectrum is unique in its specificity to mycobacteria, including atypical species important in humans such as MAC, *M. kansasii*, and the fast growers *M. fortuitum* and *M. abscessus*. This antimycobacterial-specific spectrum differs from that of isoniazid, which has very poor activity against MAC. The clinical use of R207910 will be highly targeted to the treatment of TB and mycobacterial infections.

The target and mechanism of action of R207910 are different from those of other anti-TB agents. Inhibition of ATP synthase function may lead to ATP depletion and imbalance in pH homeostasis, both contributing to decreased survival (*16*, *17*). A comparison of the sequences of ATP synthases of different bacteria and of eukaryotic ATP synthase provides a rationale for the specificity of the antibacterial spectrum and, to a lesser extent, the safety profile of R207910. Furthermore, the distinct target of R207910 means that there is no cross-resistance with existing anti-TB drugs, and our studies verified that R207910 is as effective against MDR strains as it is against fully antibiotic-susceptible strains.

R207910 has potent early bactericidal activity in the nonestablished infection murine TB model, matching or exceeding that of isoniazid (Fig. 4A), which is consistent with in vitro observations pointing to a bactericidal activity. Interestingly, the bactericidal potency seen upon administration of a single dose of R207910 (100 mg/kg) in vivo was similar to the effect seen upon continuous exposure of cultures to 0.6 μg/ml (or 10 times the median MIC of the H37Rv strain) in vitro, giving a first indication of the pharmacodynamics of R207910.

R207910 has potent late bactericidal properties in the established infection murine TB model. When given as monotherapy, the bactericidal effect of R207910 exceeds that of the reference compound rifampin, especially during the second month of therapy (Fig. 4B). Substitution of each of the three first-line drugs with R207910 resulted in a significant ($P < 0.0018$) increase in potency, leading to complete culture conversion of the lungs in some animals after only 2 months of treatment. Mouse studies to assess culture-positive relapse after follow-up without therapy are needed to clarify the sterilizing properties of R207910 in combination with other first-line agents and its potential to shorten duration of therapy. The extent of bacterial load, the most effective drug combinations, and the treatment duration needed for sterilization are all similar in mice and humans (*18*).

The extended effect of a single dose of R207910 seems to derive from a combination of the observed long plasma half-life, high tissue penetration (especially in the target organs for TB), and long tissue half-life. These are all attributes that are valuable for treatment of chronic infections, and they may also be important for the development of simpler dosing regimens.

Human studies have shown good tolerability, at least during a limited exposure period, with plasma levels ~8 times those associated with potent in vivo activity in mice. Human pharmacokinetics of R207910 seem to reflect the good oral absorption and sustained plasma levels seen in mice. The combination of low MIC values, a distinct mechanism of action, early and late bactericidal activity, and pharmacokinetic profile makes R207910 a promising TB drug candidate. Conducting clinical development in patients with active pulmonary TB is highly warranted.

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Supporting Online Material

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Materials and Methods
Supporting Online Text
Tables S1 to S8
Figs. S1 and S2

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Time-Domain Measurements of Nanomagnet Dynamics Driven by Spin-Transfer Torques

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We present time-resolved measurements of gigahertz-scale magnetic dynamics caused by torque from a spin-polarized current. By working in the time domain, we determined the motion of the magnetic moment throughout the process of spin-transfer–driven switching, and we measured turn-on times of steady-state precessional modes. Time-resolved studies of magnetic relaxation allow for the direct measurement of magnetic damping in a nanomagnet and prove that this damping can be controlled electrically using spin-polarized currents.

Spin-polarized electrons traversing a ferromagnet can transfer spin-angular momentum to the local magnetization, thereby applying a torque that may produce magnetic reversal or steady-state precession (*1*, *2*). This spin-transfer mechanism allows nanomagnets to be manipulated without magnetic fields, and it is the subject of extensive research for applications in nonvolatile memory, programmable logic, and microwave oscillators (*3*–*11*). However, the gigahertz-scale magnetic dynamics that can be driven by spin transfer have previously been measured using only frequency-domain techniques (*12*–*16*). Here we report direct time-resolved studies of dynamics excited by spin-transfer torques. By working in the time domain, we are able to characterize the full time-dependent magnetic response to pulses of spin-polarized currents, including transient dynamics. These measurements allow a direct view of the process of spin-transfer–driven magnetic reversal, and they determine the possible operating speeds for practical spin-transfer devices. The results provide rigorous tests of theoretical models for spin transfer (*1*, *9*, *17*–*19*) and strongly support the spin-torque model (*1*, *18*) over competing theories that invoke magnetic heating (*9*, *20*).

We studied nanopillar-shaped samples consisting of two 4-nm-thick permalloy (Py ≡ $Ni_{80}Fe_{20}$) ferromagnetic layers separated by an 8-nm-thick Cu spacer layer (Fig. 1A, inset). Both Py layers and the Cu spacer were etched to have an elliptical area of approximately 130 × 60 nm. Current flows perpendicular to the layers through Cu electrodes. The relative angle between the magnetic moments of the Py layers was detected by changes in the sample resistance due to the giant magnetoresistance effect. For time-resolved measurements on subnanosecond scales, signal-to-noise considerations require averaging over multiple signal traces. If the signal is oscillatory, the phase of the oscillations has to be the same in each trace or else the signal will be lost in averaging (*21*). This requires that the samples be engineered so that the initial (equilibrium) angle between the magnetic moments of the two layers, θ_0, is different from zero and is well controlled. Our devices were specially designed to provide this control. The equilibrium orientation of our top free-layer Py moment was governed primarily by the shape anisotropy of the elliptical device. We exchange-biased the bottom layer at an angle of 45° to the top layer easy axis using an 8-nm-thick antiferromagnetic $Ir_{20}Mn_{80}$ underlayer. From the combined effects of exchange biasing, shape anisotropy, and the interlayer dipole interaction, we estimated that $\theta_0 \approx 30°$. The existence of a nonzero equilibrium angle was reflected in the nonmonotonic dependence of the sample resistance (R) on magnetic field applied along the exchange bias direction (Fig. 1A for sample 1). To minimize thermal fluctuations of the initial magnetic-moment angles, we worked at low temperature (≤40 K) in a variable-temperature probe station.

The dependence of R on bias current I was similar to results from previous experiments (*7*, *13*). For magnetic field H less than the coercive field H_c of the free layer, spin transfer from I drives hysteretic switching of

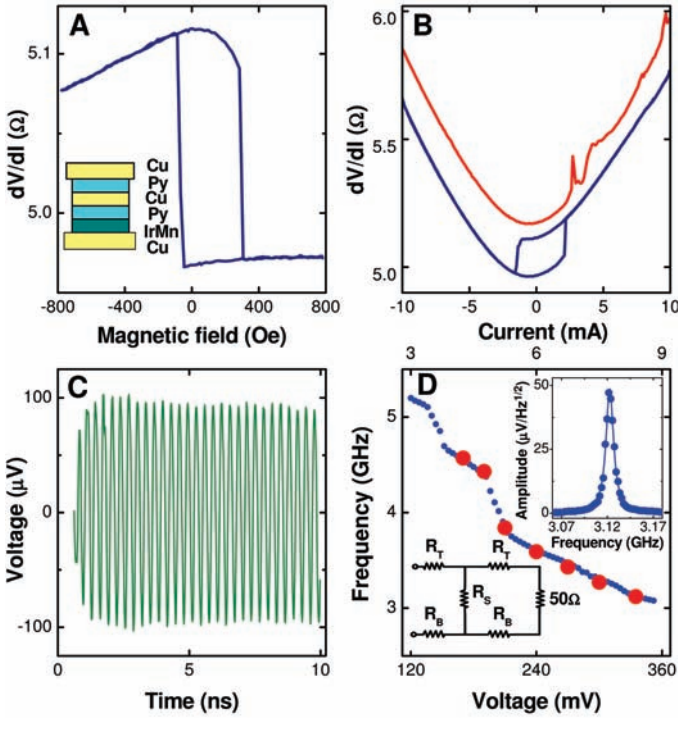

Fig. 1. Data for sample 1. (**A**) Resistance, measured in a four-point configuration, as a function of magnetic field applied along the exchange-bias direction at T = 40 K. (Inset) Composition of the sample. (**B**) Resistance as a function of current at $H = 0$ (blue line) and 700 Oe (red line, offset by 0.2 ohm). (**C**) Oscillatory voltage generated by precessional motion of the free magnet in response to a 335-mV dc voltage step applied to the device at H = 630 Oe. The data were obtained by averaging over 2 × 10^4 oscilloscope traces using the analysis described in (*23*). The start time corresponding to the midpoint of the applied bias step is t = 0.3 ns. The precessional dynamics exhibit a subnanosecond turn-on time and a long dephasing time (~10^2 ns). (**D**) Variation of the precessional frequency measured in the frequency domain with a dc bias current (blue circles, top scale) and measured in the time domain with a fast voltage step (red circles, bottom scale). (Upper inset) Frequency-domain measurement of the voltage spectrum for a dc bias I = 8.4 mA, equivalent to the voltage step amplitude in (C). (Lower inset) Equivalent circuit seen by the incident voltage step, with sample resistance R_S = 5 ohm, top contact resistances R_T = 10 ohm, bottom contact resistances R_B = 7 ohm, and the 50-ohm connection to the sampling oscilloscope.

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the free layer, and for $H > H_c$, nonhysteretic peaks and shoulders appear in the differential resistance dV/dI (Fig. 1B). Positive current corresponds to electrons flowing from the free to the pinned magnet. Frequency-domain measurements made using a spectrum analyzer demonstrate that the nonhysteretic features are associated with the turning on and off of dynamical magnetic modes and that precessional dynamics can also precede switching for $H < H_c$ (*13*, *14*, *22*).

For time-resolved measurements of spin-transfer–driven dynamics, we applied voltage steps with 65-ps rise times using a 50-GHz probe, causing steps of current through the device. Starting with the two magnetic moments in the low-resistance configuration ($\theta_0 < 90°$), steps of positive polarity applied a torque, which rotated the free-layer moment away from the fixed-layer moment and, consequently, excited free-layer dynamics (*1*, *7*). We monitored the voltage across the device with another 50-GHz probe connected to a 12-GHz sampling oscilloscope through a 25-dB amplifier. The measured signal is the sum of a voltage step transmitted through the device and an oscillatory signal due to magnetic dynamics. The signal caused by magnetic dynamics was determined by a background-subtraction procedure (*23*).

Figure 1C shows the magnetic-dynamics component of the transmitted voltage for a 335-mV incident step at H = 630 Oe. A strong oscillatory signal is seen at 3.12 GHz. This frequency increases with H as expected for a signal due to precession of magnetic moment in a field and decreases with increasing amplitude of the voltage step. A similar dependence of the steady-state precession frequency on field and dc current is found in frequency-domain measurements, so we can calibrate our current step amplitudes by comparing the two types of experiments (Fig. 1D). [Discontinuities in Fig. 1D are related to transitions between precessional modes (*15*)]. The conversion factor between the incident voltage amplitude and the current through the sample is 0.025 ohm^{-1}. This calibration agrees with the equivalent circuit for our apparatus (Fig. 1D, inset) to within 5%.

The amplitude of the oscillatory signal in Fig. 1C increases from zero to a maximum over a subnanosecond time interval, followed by a slow decay. Because frequency-domain measurements show that the motion at long times is a steady-state precessional mode, the decay should not be understood as an actual loss of precession amplitude. Instead, it is an effect of averaging over multiple traces that do not maintain phase coherence. The measured dephasing rate (~10 MHz) corresponds well to the width of the spectral peak in the frequency domain at the equivalent dc bias (Fig. 1D, inset). For the bias conditions of Fig. 1, C and D (inset), the amplitudes of the signals in both the time- and frequency-domain measurements are consistent with a maximum precession angle of approximately 35° (*23*).

The turn-on time of the oscillations (<1 ns) in Fig. 1C is much faster than the dynamics excited in samples for which the moments of the two magnetic layers are initially approximately parallel (*21*). This confirms simulations showing that an increasing offset angle θ_0 should reduce this response time (*18*, *24*).

At $H < H_c$, spin transfer can drive magnetic reversal. The dynamical process by which reversal is achieved has been a subject of considerable theoretical debate. Some models (*1*, *18*) predict that the transfer of angular momentum can generate precession in a nanomagnet with a precession angle that increases continuously until the moment has flipped. Other models (*9*, *20*) have suggested that spin transfer excites short-wavelength magnons, effectively heating the sample incoherently to accelerate reversal. Our time-domain techniques enabled us to observe directly the dynamics throughout the process of spin-transfer–driven reversal, and we found that reversal on nanosecond scales was accomplished by a process of precession. For this measurement, we applied two pulse sequences (signal and baseline) at H = 0 Oe (*21*). For the signal sequence, we began with the two magnetic moments in the low-resistance state and then applied a 10-ns current pulse of positive polarity, followed by a 100-ns reset current pulse of negative polarity and a 10-μs waiting time at zero bias. The nanomagnet switched from the low- to the high-resistance state during the positive-going pulse of this sequence and was returned to the low-resistance state by the reset pulse. To obtain a baseline signal for subtracting the transmitted voltage pulse, we started with the moments in the high-resistance configuration and applied only 10-ns pulses of positive polarity, so that the moments always remained in the high-resistance state.

The signal-minus-baseline traces are shown in Fig. 2, A to C, for three magnitudes of the current pulse. The initial voltage drop V is due to the pulse onset at time $t \approx 0.3$ ns. After the pulse onset, coherent oscillations are present, which lead to an increase in V that corresponds to reversal from the low- to high-resistance magnetic configuration. The transition between the two resistance states in Fig. 2, A to C, appears to be gradual. However, we argue that this apparent gradualness can be explained by averaging over multiple switching events, each with a sharp transition but with a distribution of initial magnetic-orientation angles due to thermal fluctuations and possibly different initial micromagnetic configurations (*25*). Figure 2D shows the results of a simulation of current-induced nanomagnet switching performed by numerical solution of the Landau-Lifshitz-Gilbert (LLG) equation of motion, including the Slonczewski form of the spin-transfer torque (*26*), solved in the approximation that the nanomagnet responds as a single macrospin. The blue line in Fig. 2D shows the calculated voltage trace for an individual

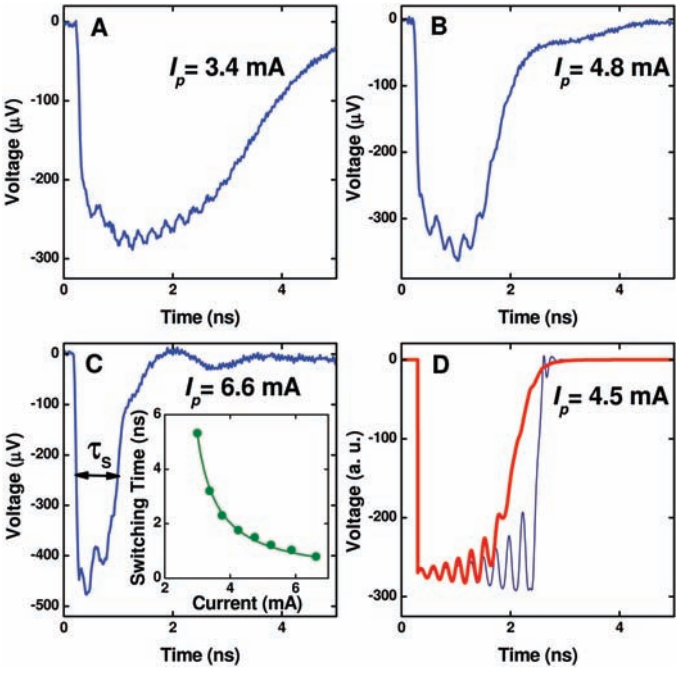

Fig. 2. Voltage signals due to current-induced switching of the free-layer magnet in sample 1 at H = 0 Oe and T = 40 K, during a 10-ns current pulse of three different amplitudes: (**A**) I_p = 3.4 mA, (**B**) I_p = 4.8 mA, and (**C**) I_p = 6.6 mA. Each plot represents an average over 4 × 10^4 oscilloscope traces. [Inset in (C)] Dependence of switching time on I_p, with a fit to $\tau_S = \tau_A/(I_p/I_0 - 1)$. (**D**) Voltage signals given by LLG simulations of current-induced switching, assuming $\mu_0 M_S$ = 0.81 T for Py, a damping parameter α = 0.025, a current polarization P = 0.3, a uniaxial anisotropy field = 300 Oe, and a 30° angle between the magnetic moment of the free layer and the current polarization. The blue line shows an individual switching event. The red line is an average over 2000 switching events with a Gaussian distribution of initial directions for the magnetic moment equivalent to thermal fluctuations at 40 K. a.u., arbitrary units.

event of precessional reversal. The red line is an average over 2000 switching events for a Gaussian distribution of the initial angle for the moment of the free magnet. We assume a standard deviation of 4°, corresponding to the thermal fluctuations at temperature $T = 40$ K for a nanomagnet with the uniaxial anisotropy energy of 0.8 eV. Given the quality of agreement between the simulations and the data in Fig. 2, A to C, we conclude that phase-coherent precessional motion within the spin-torque model gives a good description of the current-induced switching process.

We define the switching time τ_S as the interval between the pulse onset and the midpoint of the transition between the two resistance states. The dependence of τ_S on the amplitude of the current pulse I_p is plotted in the inset of Fig. 2C. The data are fit well by the expression $\tau_S = \tau_A/(I_p/I_0 - 1)$ predicted by LLG simulations of spin-torque–induced reversal (*18*), with the zero-temperature critical current for nanomagnet switching $I_0 = 2.37 \pm 0.03$ mA and $\tau_A = 1.39 \pm 0.06$ ns, where τ_A is a fitting parameter.

One of the fundamental predictions of the spin-torque model is that small applied spin-polarized currents should modify the effective magnetization-damping parameter α' of a nanomagnet (*1*). According to this model, the spin-transfer torque has a large component that is colinear with the Gilbert damping torque. Therefore, the spin torque can act to enhance or reduce the effective damping, depending on the sign of I. At the critical current for the onset of dynamical states, the effective damping is expected to go to zero for small-angle excitations. This prediction has not been tested previously because the damping parameter cannot be determined from frequency-domain spectra such as those shown in Fig. 1D. However, by exciting the free-layer moment to a nonequilibrium angle and watching in the time domain as it relaxes to equilibrium, we can directly measure the effective magnetic damping as a function of I.

The minimum current required to excite magnetic dynamics at $H = 0$ Oe for sample 2 is $I_D = 1.25 \pm 0.05$ mA, based on frequency-domain measurements of the microwave signal emitted by the precessing magnet under dc bias (Fig. 3A). To measure the dependence on I of the decay time for magnetic relaxation, τ_d, and thus of the effective damping α', we began with the device in the low-resistance state at $H = 0$ Oe, biased it with a subcritical ($I \leq I_D$) positive dc current, and then applied a positive 3-mA 650-ps pulse. From measurements such as those shown in Fig. 2, A to C, we determined that this short pulse rotates the free-layer magnetic moment away from its equilibrium direction but does not produce magnetic reversal. Therefore, after the falling edge of the pulse, the moment relaxes back to equilibrium by magnetic precession with a decreasing amplitude, producing (because of the current bias) a decaying oscillatory voltage. For background determination, we repeated the measurement with the sample initially in the high-resistance configuration and subtracted the result from the signal traces. Figure 3B shows the magnetic relaxation signal for a 0.6-mA dc bias current. By fitting to an exponentially decaying sine function, we determined a relaxation time of 0.85 ns. This is much shorter than the dephasing time for the regime of steady-state precession, indicating that the decaying signal corresponds to an oscillation with decreasing amplitude rather than a loss of phase coherence. As we increased I toward I_D, the relaxation time grew markedly (Fig. 3C). In Fig. 3D, we summarize the results for several values of I in terms of the effective damping parameter $\alpha' = 2/(\tau_d \gamma \mu_0 M_S)$ (*27*), where γ is the gyromagnetic ratio, μ_0 is the magnetic permeability of free space, M_S is the free-layer magnetization, and $\mu_0 M_S = 0.81$ T as measured by superconducting quantum interference device (SQUID) magnetometry for a 4-nm Py film. The damping exhibits an approximately linear decrease with increasing I, extrapolating to zero near I_D, in excellent agreement with the prediction of the spin-torque model (*1*, *18*). The measured damping does not go to zero precisely at I_D, probably because our measurements involve tipping angles that are larger than the steady-state precession angle for the dynamical modes at I just above I_D. If we extrapolate the measured effective damping to $I = 0$, we estimate the damping parameter of the free magnet to be $\alpha = 0.025$, larger than the value $\alpha = 0.007$ obtained from ferromagnetic resonance measurements of a 3-nm-thick Py film between Cu layers (*28*). Our larger value may be due to spin pumping between the two magnetic layers in our device (*29*, *30*) and/or edge effects at the boundary of the nanopillar.

Our time-resolved measurements have important implications both for the theoretical understanding of spin-transfer torques and for potential applications. All of our data are in excellent agreement with the predictions of the Slonczewski spin-torque model (*1*): that spin transfer can reduce the effective magnetic damping for applied currents less than a critical current and, at higher currents, can drive phase-coherent precessional magnetic dynamics. The measurements are not consistent with models in which the dominant spin-transfer mechanism is incoherent magnon excitation equivalent to effective heating (*9*, *20*). Spin transfer can drive magnetic reversal by precession, with switching times that are less than 1 ns and exhibit narrow statistical distributions, which are very promising characteristics for magnetic-memory applications. For larger applied magnetic fields, the steady-state precessional modes produced by spin transfer can have short turn-on times (<1 ns) and long decoherence times ($\sim 10^2$ ns), which are appropriate for applications in high-speed signal processing.

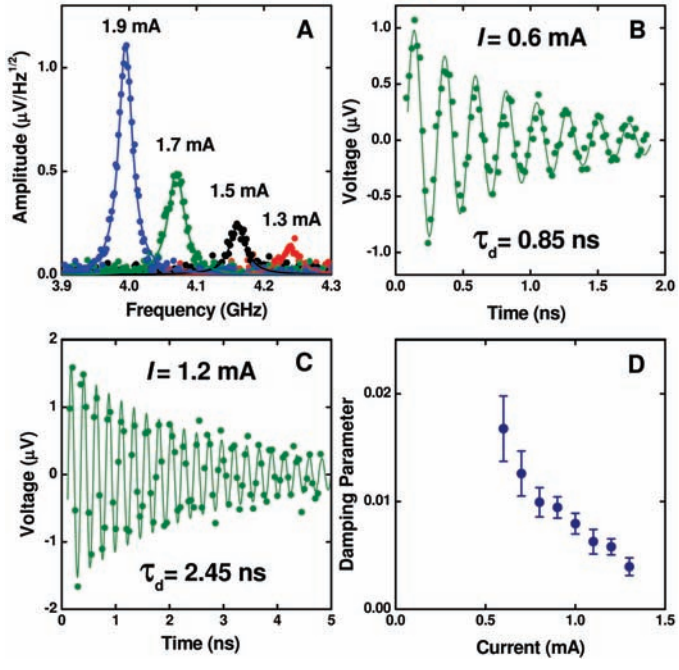

Fig. 3. Data for sample 2. (**A**) Frequency spectra of magnetic precession signals that are generated under different dc current biases at $H = 0$ Oe and $T = 7$ K. The extrapolated critical current for the onset of precessional dynamics is $I_D = 1.25 \pm 0.05$ mA. (**B** and **C**) Signals generated by a free decay of the nanomagnet to its equilibrium direction in the presence of currents $I < I_D$: (B) $I = 0.6$ mA and (C) $I = 1.2$ mA. The data in (B) and (C) were obtained by averaging over 10^6 oscilloscope traces. The start time corresponding to the midpoint of the step to the final current value is $t = -0.1$ ns. The lines are fits to exponentially decaying sinusoid functions. (**D**) Dependence of the effective damping parameter α' on dc bias current. Error bars indicate the 68% confidence limits obtained from the least-squares fit to the oscillatory decay data such as shown in (B) and (C).

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Al Cluster Superatoms as Halogens in Polyhalides and as Alkaline Earths in Iodide Salts

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Two classes of gas-phase aluminum-iodine clusters have been identified whose stability and reactivity can be understood in terms of the spherical shell jellium model. Experimental reactivity studies show that the $Al_{13}I_x^-$ clusters exhibit pronounced stability for even numbers of I atoms. Theoretical investigations reveal that the enhanced stability is associated with complementary pairs of I atoms occupying the on-top sites on the opposing Al atoms of the Al_{13}^- core. We also report the existence of another series, $Al_{14}I_x^-$, that exhibits stability for odd numbers of I atoms. This series can be described as consisting of an $Al_{14}I_3^-$ core upon which the I atoms occupy on-top locations around the Al atoms. The potential synthetic utility of superatom chemistry built upon these motifs is addressed.

The electronic properties and chemical reactivity of small metal clusters can be fundamentally different from bulk metals, which are well described by band theory, and compounds with only a few metal atoms, where bond formation and charge states typically lead to stable atomic shells for each atom. For clusters of free-electron metals, a model is commonly used in which the nuclei and innermost electrons form a positively charged core with an essentially uniform potential. All of the valence electrons from the individual atoms in the cluster are then subjected to this potential, and the jellium electronic shell structure emerges with stable configurations of electrons (2, 8, 18, 20,…) that differ from the atomic series (2, 10, 18,…) (*1*–*6*).

Such clusters could be described as superatoms, because clusters of a given element can have chemical and electronic properties resembling those of another atom; hence, superatoms can be regarded as an extension of the periodic table to a third dimension (*1*–*23*). Similar principles have been discussed for the description of the properties of various functional groups in synthetic chemistry, particularly Grimm's hydrogen-displacement theorem (pseudoelements) and Haas' concept of paraelements (*24*).

We recently demonstrated that an Al_{13} cluster acts like a superhalogen even when combined with a conventional halogen, namely I, to form an $Al_{13}I^-$ cluster compound (*22*, *23*). Through reactivity studies of Al_nI^- clusters combined with state-of-the-art ab initio calculations, we find that $Al_{13}I^-$ is a remarkably stable species and that the extra electron charge in $Al_{13}I^-$ is mostly localized at the Al_{13} unit.

Halogens form extended polyhalides in both the gas and condensed phases. For example, I forms polyiodides within the series I_{2n+1}^- that consist of I^- or I_3^- ions bound to I_2 molecules in the form of chains. As an example, the ground state of I_5^- is a V-shaped structure with two I_2 molecules coordinated to an apical iodide $[(I^-)\bullet 2I_2]$. Given that Al_{13}^- can act as a halide ion when coordinated to one or two I atoms, one would predict that interactions with I_2 molecules might lead to complex clusters with structures similar to I_{2n+1}^- polyiodides.

Herein, we present evidence of the formation of a previously unknown class of polyhalide-like molecules by replacing an I atom in traditional polyiodides with an Al_{13}^- cluster. Although it is possible to form $Al_{13}I_x^-$ clusters for all x, trends in reactivity reveal that members of this series are particularly stable when x is even. Because traditional interhalogen and polyhalide ions require an odd total number of halogen atoms, the $Al_{13}I_x^-$ clusters appear to resemble these known species. Our ab initio calculations, however, indicate that this apparent similarity is slightly deceptive and that the $Al_{13}I_{2x}^-$ clusters present an entirely different geometry with subtle differences in chemical behavior. Whereas polyiodides contain I_2 molecules, superpolyhalides only contain I atoms. Further, the stability of clusters with even numbers of I atoms has a completely different origin than the stability in conventional polyiodides.

We also show that Al_{14} can behave as an alkaline earth metal–like superatom. A second series of clusters of the type $Al_{14}I_x^-$ exhibits pronounced stability when x is odd. The series begins with $Al_{14}I_3^-$, in which the Al_{14} core behaves as a dication, and from our theoretical investigations we describe the mechanism by which larger members of the series build upon this core. The synthetic importance of these gas phase findings are underscored by recent results that show that the solution phase "metalloid" cluster compounds (*25*, *26*) synthesized by Schnöckel and co-workers are in fact, at their core, the same cluster species found in the gas phase (*27*, *28*).

The Al cluster anions react readily with I_2 (Fig. 1 and fig. S1), leading to a distribution of clusters of the type $Al_nI_x^-$ (*29*). Despite the addition of I_2, x need not be even. The addition of single I atoms might be partially attributed to the equilibrium of I_2 with atomic I in the vapor phase, but, as we describe in more detail

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below, etching reactions that lead to AlI or AlI_3 formation are likely the primary source of $Al_nI_x^-$ clusters. At very low concentrations of I_2, a broad distribution of peaks, which includes the $Al_{13}I^-$ peak, is evident (fig. S1). Even in this concentration regime, however, $Al_{13}I_2^-$ is more abundant. At higher concentrations (Fig. 1B), a distinct pattern emerges, featuring a pair of peaks spaced 27 atomic mass units (amu) from each other and repeating in intervals of 127 amu. These correspond to $Al_{13}I_x^-$ and $Al_{14}I_x^-$. The $Al_{14}I_x^-$ series begins at $x = 3$, and the $Al_{14}I_3^-$ cluster was actually observed previously as a product of the reaction between Al_n^- and HI (*22*, *23*). In the present experiments, the more intense peak in each 13-14 pair alternates at each value of x. When x is even, the $Al_{13}I_x^-$ clusters dominate, and when x is odd the $Al_{14}I_x^-$ clusters dominate. This evidence alone suggests that the chemical stability of the two-cluster series is dependent on the odd-even character of x. Most importantly, however, this trend is confirmed when the iodized clusters undergo oxygen etching (Fig. 1C): The minor peak in each pair is substantially diminished, and the major peak persists. $Al_{13}I_x^-$ clusters are only stable when x is even, whereas $Al_{14}I_x^-$ clusters require that x is odd.

In our previous report on Al_{13}'s superhalogen character (*22*), the stability of $Al_{13}I^-$ was explained in terms of the ability of the halogen-like Al_{13} cluster moiety to retain its preferred geometric, electronic, and charge properties. In the present case, the same principles apply, but surprising intricacies emerge. If the I atoms were simply a source of electrons, then we would expect the stability of $Al_{13}I_x^-$ to increase with x without any dependence on odd or even values. The oxygen-etching experiments indicate that this is not the case. Simply providing the Al_{13} cluster with electron-rich neighbors proves an inadequate chemical scenario for the emergence of special stability.

To further probe the origin of the stability of these complexes and the nature of electronic interactions, we carried out theoretical calculations with the use of a first-principles molecular orbital approach wherein the cluster wave function is expressed as a linear combination of atomic orbitals centered at the atomic sites (*30*–*34*). The exchange correlation corrections were included within a gradient-corrected density functional (*34*). The ground state of Al_{13}^- is an icosahedral structure with an Al-Al bond length of 2.80 Å between the surface sites. For a single I, the ground state of $Al_{13}I^-$ corresponds to an almost-perfect icosahedral Al_{13} moiety with I occupying an on-top site (Fig. 2). An analysis of the charge density of the highest occupied molecular orbital (HOMO) (Fig. 2) reveals that most of the charge of the additional electron is localized at the Al_{13} moiety, thus suggesting superhalogen character and the possibility of polyhalide generation. The occupation of the on-top site by I generates charge localization on the Al vertex opposite from the I atom. These regions of localized charge are termed active centers.

We recently examined structures for $Al_{13}I_2^-$ in which an I_2 molecule was placed in several orientations around a surface Al site or where a second, dissociated I occupied various Al sites different from the initial I occupation on Al_{13}^- (*23*). In each case, the geometry was optimized by moving atoms in the direction of forces until the forces dropped below a threshold value of 0.001 atomic units. After an investigation of these geometries and possible spin multiplicities, we found that the ground state (Fig. 2) corresponds to an I atom occupying the on-top site opposite from the first I atom, where the active center on $Al_{13}I^-$ was observed. In terms of polyhalide-like clusters, Al_{13}^- and $Al_{13}I_2^-$ can be considered as analogous to the Br^- and BrI_2^- building blocks discussed previously (*22*). We extended the studies to all $Al_{13}I_x^-$ (x values from 3 to 12) by successively adding an I atom and examining all possible positions (including structures more closely related to the branching chains found in polyhalides, which were found to be less energetically stable) and spin multiplicities. In Fig. 2, we show the ground-state geometries and the

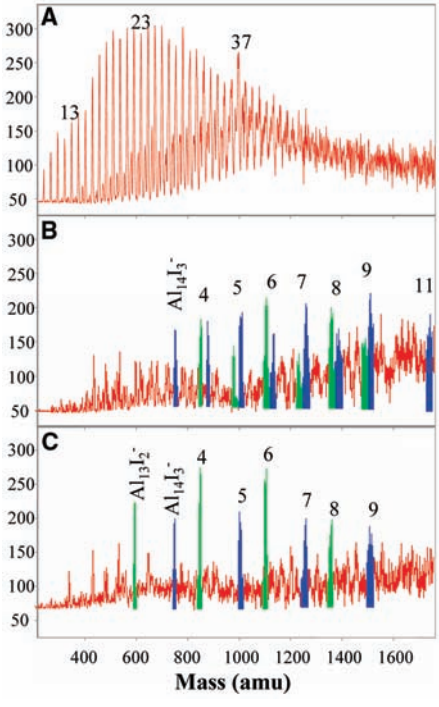


Fig. 1. Mass spectra of (**A**) Al cluster anions (**B**) reacted with I_2 vapor and then (**C**) etched by O_2. Peaks shaded green fall into the $Al_{13}I_x^-$ family, whereas peaks shaded blue fall into the $Al_{14}I_x^-$ family. In all panels, the y axis is peak intensity (in arbitrary units).

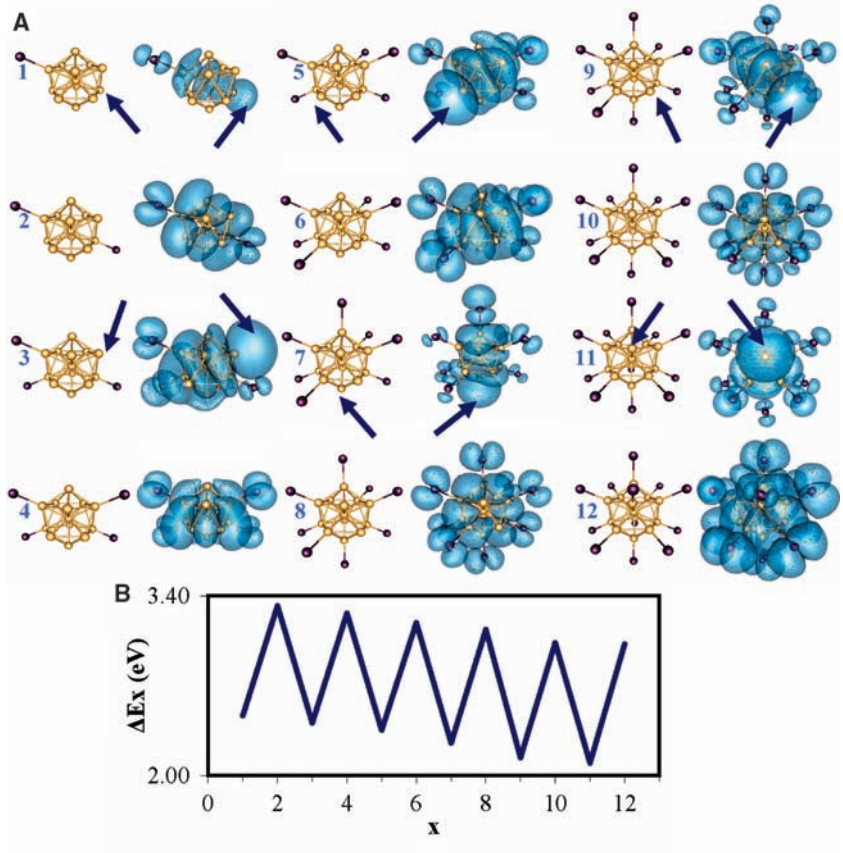


Fig. 2. (**A**) Lowest energy structures and charge maps for $Al_{13}I_x^-$ (x values from 1 to 12). The areas of high charge density, or active sites, are indicated by arrows. (**B**) ΔE_x (Eq. 1), the energy to remove one I atom from $Al_{13}I_x^-$ for x values from 1 to 12.

charge density of the corresponding HOMO levels. All of the $Al_{13}I_x^-$ clusters with odd x are marked by the presence of active centers, whereas in the clusters with even x a pair of I atoms occupy opposing sites, thus filling the active site generated by the previous (odd) I. A careful look at the HOMO charge density reveals another interesting feature. In the clusters with odd x, the bonding to Al_{13} is reminiscent of a σ bond, whereas in those with even x the bonds look π-like, indicating a change in bonding between a single I or a pair to an orbital of Al_{13}. In all of the $Al_{13}I_x^-$ clusters, the Al moiety has an almost perfect icosahedral geometry, just as in Al_{13}^-.

Our theoretical studies can account for the greater stability of clusters with even numbers of I atoms as shown in the plot of the change in energy (ΔE_x)

$$\Delta E_x = -E(Al_{13}I_x^-) + E(Al_{13}I_{x-1}^-) + E(I) \quad (1)$$

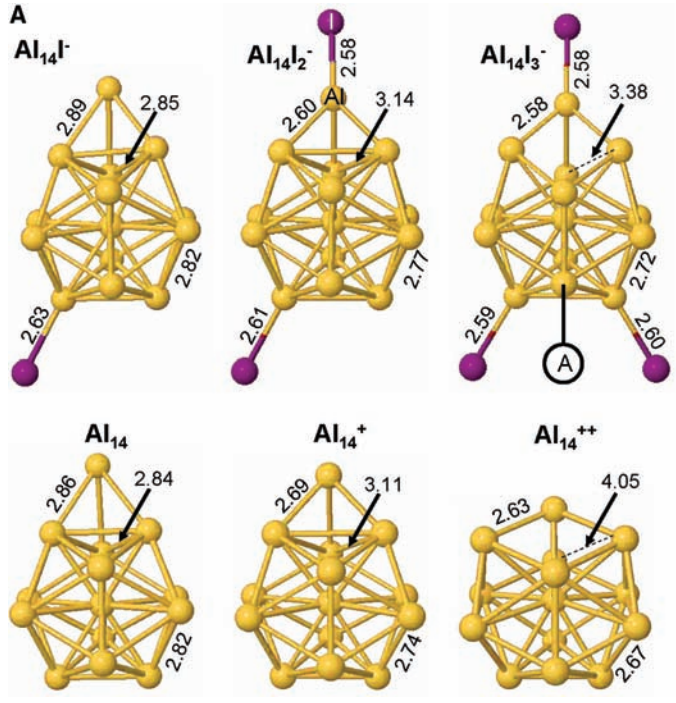
which represents the gain in energy as successive I are added (Fig. 2B). Here, $E(Al_{13}I_x^-)$ is the total energy of a cluster of 13 Al and x I atoms. The ΔE_x for clusters with odd x is around 0.9 eV (20.8 kcal mol^{-1}) less than the value for clusters with even x, which indicates that the clusters with even x are particularly stable.

As a further probe of the active center hypothesis, we performed experiments whereby $Al_nI_x^-$ clusters were first formed by reaction with I_2 and then reacted with methyl iodide (CH_3I). Previously, we found that Al_{13}^- was unreactive toward CH_3I (*23*, *35*). In the present experiments, $Al_{13}I_x^-$ clusters with odd x were quite reactive toward CH_3I, but those with even x were substantially less reactive (*36*). Recall that the polyhalides also exhibit enhanced stability for I_n^- with odd n. There, the stability is associated with the presence of I_2 molecules, because the unit can be expressed as $X^-{\cdot}(I_2)_n$ (X indicates I^- or I_3^- for polyiodides or, for example, Br^- for an interhalogen). In the case of replacement of an I atom by the Al_{13} superatom, the odd-even trend in stability has a completely different electronic origin. These interhalogen molecules represent a new class of superatom-containing molecules with distinctive chemical properties.

As in many cluster systems, the addition or removal of one I atom has a profound effect on the stability of $Al_{13}I_x^-$ clusters. The present experiments show that, as we would expect, the system is also extremely sensitive to the number of Al atoms. We now address the emergence of the prominent $Al_{14}I_x^-$ series. The previous observation of $Al_{14}I_3^-$ as a product in the HI etching experiments (*22*, *23*), and the fact that this cluster is the first member of the $Al_{14}I_x^-$ series in the present study, strongly indicate that it represents the core upon which the larger clusters are built. The behavior of this core is also explainable in terms of the spherical shell model and the superatom concept. Because Al_{14} has a lower electron affinity than I, one could imagine that each I added to the cluster will lead to the withdrawal of one electron. Because Al_{14}^- has 43 valence electrons, three I atoms will be needed to recreate a 40-electron core that could serve as the foundation upon which larger clusters are built. Theoretical studies are critical to the confirmation of this conjecture. For the superatom description to provide an accurate representation of this system, one would expect the Al_{14} core in $Al_{14}I_x^-$ clusters to mimic a free Al_{14} in $Al_{14}I^-$, a free Al_{14}^+ in $Al_{14}I_2^-$, and a free Al_{14}^{2+} in $Al_{14}I_3^-$.

Credence is given to the assignment of an Al_{14}^{2+} core to $Al_{14}I_x^-$ by considering similarities between the ground-state geometries of the $Al_{14}I_x^-$ series and free Al_{14}, Al_{14}^+, and Al_{14}^{2+} (Fig. 3). The structure of Al_{14} can be regarded as an extra Al atom bound to the hollow site of three Al atoms occupying a triangular face of the Al_{13} icosahedron. Bond lengths between the extra (on-top) Al and Al atoms of the triangular face, as well as bond lengths between cotriangular-face Al atoms, follow the same bond length trends in both the iodized and free cluster series. Bond lengths between the Al atoms of the triangular face increase continuously with the oxidation of Al_{14} as with the addition of I to $Al_{14}I^-$. The Al-Al bonds are ultimately broken, comparably, in Al_{14}^{2+} and in $Al_{14}I_3^-$. Furthermore, the broken Al-Al bond length is larger in free Al_{14}^{2+} than in $Al_{14}I_3^-$, as expected because of reduction of the Coulomb repulsion with the addition of I to the free cluster. Additionally, the overall shape of the Al_{14} unit becomes increasingly spherical as it is charged. These systemic trends support the description of $Al_{14}I_x^-$ clusters within the spherical shell jellium model.

To further confirm the resemblance of the Al_{14} core of $Al_{14}I_3^-$ to free Al_{14}^{2+}, we first superimposed the charge density of a free Al_{14}^{2+} cluster and three free neutral I atoms positioned to conform to the geometric configuration of $Al_{14}I_3^-$. This superimposed charge density was then subtracted from the charge density of $Al_{14}I_3^-$ to determine the localization of the $Al_{14}I_3^-$ cluster's three additional



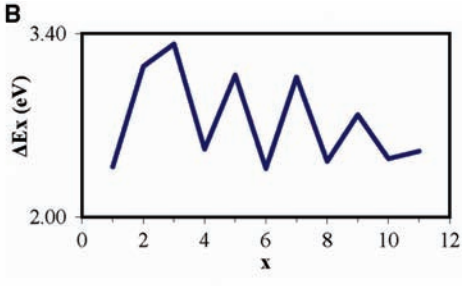


Fig. 3. (**A**) Lowest energy structures for $Al_{14}I_x^-$ and for $Al_{14}^{(x-1)+}$. The Al_{14} moieties in the iodized clusters where $x > 2$ more closely resemble the dication. (**B**) ΔE_x, the energy to remove one I atom from $Al_{14}I_x^-$ for x values from 1 to 12.

electrons (two from Al_{14}^{2+} and one making the cluster anionic). By this method, most of the electronic charge was found to be localized at the I sites, reconfirming that the Al_{14} core indeed resembles Al_{14}^{2+}.

The theoretical studies show that the progression of larger clusters upon the $Al_{14}I_3^-$ core proceeds in an interesting manner. In $Al_{14}I_4^-$, the additional I occupies the site (marked A in Fig. 3A) of the $Al_{14}I_3^-$ base. The structure of $Al_{14}I_5^-$, however, corresponds to an $Al_{14}I_3^-$ core and the two I atoms occupying a pair of sites opposite to each other in the Al_{14} portion, leaving site A unoccupied. Thus, whereas site A is occupied in all of the clusters with an even number of I atoms, the clusters with an odd number of I atoms feature pairs of I atoms (not bound as I_2) at the Al sites opposite to each other, much in the same way as in the $Al_{13}I_x^-$ series. However, the I at site A is not as strongly bound, so the clusters with an odd number of I atoms are more stable than those with an even number of I atoms. The final manifestation of such a model comes in the variation of the energy ΔE_x (Fig. 3). Note the odd-even oscillations in energy after three I atoms.

Lastly, we consider the results of our Mulliken charge density analysis. The directionality of the cluster-I bonds switches from polar toward the Al cluster in Al_{13}-based species to polar toward the I atoms in the Al_{14}-based clusters. As described above, in the Al_{13} series the Al_{13} superhalogen cluster pulls electronic charge from the I atoms in order to create what is essentially an Al_{13}^- core. In $Al_{14}I_3^-$, the cluster-I bonds are polar in the other direction so that the majority of the anionic cluster's charge resides with the I atoms. This piece of information, combined with the preceding analyses, shows that the description of this system as a molecule containing an alkaline earth–like superatom is valid. That is, although bare Al_{14} does not lend itself to description by the spherical shell model (*37*), it can be structurally and electronically manipulated to do so in an appropriate chemical environment. Significantly, similar arguments of charge-withdrawing or -donating ligands on clusters have been advanced by other researchers, in particular, the work of Fagerquist *et al.* on $Ag_xI_y^{+/-}$ clusters (*38*, *39*) and the work of Wang and co-workers on "multiply charged" metal clusters (*40*).

We can use our theoretical treatment to understand the thermochemistry involved in our experiments. On the basis of values reported in our previous publication (*23*), we describe several of the key reaction pathways involved in the formation of $Al_{13}I_x^-$ clusters:

$$Al_{13}^- + I_2 \rightarrow Al_{13}I_2^- \quad (2)$$

$$Al_{13}^- + I_2 \rightarrow Al_{13}I^- + I \quad (3)$$

$$Al_{14}^- + I_2 \rightarrow Al_{13}I^- + AlI \quad (4)$$

According to our calculations, the reaction in Eq. 2 is energetically favorable by 3.63 eV so that, although this is the most favored reaction between Al_{13}^- and I_2, the reaction in Eq. 3, which is energetically favorable by 0.31 eV, is also possible. Moreover, from a kinetic point of view, this requires a third body for stabilization. These mechanisms are similar to those described in the reactions with HI (*22*, *23*). Equation 4 shows an example of I_2 acting as an etchant; the particular etching reaction shown here is energetically favorable by 2.25 eV. Other etching pathways may also be possible at various values of n, as shown in Eqs. 5 to 7.

$$Al_nI_x^- + I_2 \rightarrow Al_{n-1}I_{x+1}^- + AlI \quad (5)$$

$$Al_nI_x^- + I_2 \rightarrow Al_{n-2}I_x^- + 2AlI \quad (6)$$

$$Al_nI_x^- + I_2 \rightarrow Al_{n-1}I_{x-1}^- + AlI_3 \quad (7)$$

Although Eqs. 5 and 6 could be applied to the $x = 0$ case, Eq. 7 could not. The $Al_{14}I_x^-$ series must form via one or more of the etching mechanisms in Eqs. 4 to 6 from clusters with $n > 14$. The energetic treatment of Eq. 4 shows that, in contrast to the $Al_{13}I_x^-$ series, the $Al_{14}I_x^-$ series cannot arise directly via attachment of I atoms to the bare Al cluster anion. The energetics of the reactions between $Al_nI_x^-$ clusters and oxygen are less easily analyzed, but for various values of n and x, the oxygen etching reaction yields different Al oxides, I oxides, and $AlIO_2$ molecules. The relative resistance of some clusters to reaction with oxygen seems to be kinetically, rather than thermodynamically, mediated.

In a previous study (*22*), we had compared $Al_{13}I^-$ and $Al_{13}I_2^-$ to BrI^- and BrI_2^- by considering electron affinity (EA) the defining characteristic of a halogen. It seems apparent from the structural and electronic properties of the larger $Al_{13}I_x^-$ clusters that perhaps the defining characteristic in these interhalogens is the size, rather than the EA, of the "halogen." If EA were more important, branching chain structures more reminiscent of bromoiodides would emerge; instead, fluorohalide-like structures are found in which the smaller halogen atoms decorate a central larger halogen atom.

In the $Al_{14}I_x^-$ series, we have shown that the Al_{14} moiety approximates the structural and electronic character of an Al_{14}^{2+} ion. It is therefore concluded that $Al_{14}I_3^-$ serves as the fundamental core upon which larger $Al_{14}I_x^-$ clusters build because of the presence of a closed shell (40-electron) Al_{14} alkaline earth–like superatom. We have shown that, in the proper chemical environment, Al_{14} can be described by the spherical shell model. On the basis of our analysis, we predict that the stability of the Al_{14} series should follow similar trends for the other halogens, because their EA values allow them to be even more efficient as electron-withdrawing ligands. In both the $Al_{14}I_x^-$ and the $Al_{13}I_x^-$ series, the sequential addition of I atoms to the superatom core leads to the emergence and quenching of active centers. These centers should provide many opportunities to explore new cluster chemistry.

The implementation of the spherical shell jellium model allows for a sound understanding of these halogenated aluminum clusters. By extension of the superatom concept, it is possible to understand that the chemical manipulations highlighted here should apply to other metal cluster systems. Although the type of halogen chemistry that is possible with Al_{13} may not be completely universal to all superatoms with halogen-like electronic structure (the superhalogen nature of Al_{13} is quite distinctive, but Al_{23}^-, Al_{37}^-, and Al_{55}^- also have anomalously high electron affinities), the observation of superatom character for Al_{14}-based clusters, which were not previously thought to be amenable to a jellium description, suggests that the synthetic potential of superatom chemistry may completely exceed prior expectations.

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29. The current experimental studies were carried out in

a fast-flow tube apparatus (*41*) equipped with a constant-flow laser vaporization (LaVa) source. Bare Al_n^- clusters were generated via laser ablation of a translating and rotating Al rod in the presence of a constant flow [8000 standard cm^3 min^{-1} (sccm)] of high-purity He gas. The clusters were collisionally cooled to room temperature and exposed to I_2 vapor seeded in He, which was introduced through a radial-type reactant gas inlet (RGI). In order to generate a steady flow of I_2, the sublimation vessel was heated. A tandem reaction setup was also used in which I_2 vapor was introduced through one RGI and O_2 was introduced through a second RGI downstream. In this manner, the relative stabilities of the products of the $Al_n^- + I_2$ reaction could be assessed via oxygen etching. In either configuration, product and reactant clusters were sampled through a 1-mm orifice and analyzed via quadrupole mass spectrometry.

30. The calculations were carried out by using a first-principles molecular orbital approach within a density functional framework. Here, the molecular orbitals are expressed as a linear combination of atomic orbitals formed via a combination of Gaussian functions centered at the atomic sites. The exchange correlation contributions are included within a gradient-corrected density functional formalism. The actual calculations were carried out with the use of the Naval Research Laboratory Molecular Orbital Library (NRLMOL) developed by Pederson and co-workers (fig. S1) (*32*). Here, the hamiltonian matrix elements are evaluated by numerical integration over a mesh of points. The basis set for Al had 6s, 5p, and 3g Gaussians; those for I had 8s, 7p, and 5d Gaussians. The basis sets were supplemented with a d-Gaussian. For details of the codes and the basis sets, the reader is referred to earlier papers.
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42. We gratefully acknowledge financial support from U.S. Department of Energy grant DE-FG02-02ER46009 and U.S. Air Force Office of Scientific Research grant F49620-01-1-0380 for the experiments involving Al_{13}-based clusters. We also thank M. L. Kimble for contributions to the experimental work.

Supporting Online Material

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Fig. S1

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A Stable Aminyl Radical Metal Complex

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Metal-stabilized phenoxyl radicals appear to be important intermediates in a variety of enzymatic oxidations. We report that transition metal coordination also supports an aminyl radical, resulting in a stable crystalline complex: $[Rh(I)(trop_2N^{\bullet})(bipy)]^+OTf^-$ (where trop is 5-H-dibenzo[a,d]cycloheptene-5-yl, bipy is 2,2′-bipyridyl, OTf^- is trifluorosulfonate). It is accessible under mild conditions by one-electron oxidation of the amide complex $[Rh(I)(trop_2N)(bipy)]$, at a potential of −0.55 volt versus ferrocene/ferrocenium. Both electron paramagnetic resonance spectroscopy and density functional theory support 57% localization of the unpaired spin at N. In reactions with H-atom donors, the Rh-coordinated aminyl behaves as a nucleophilic radical.

Free aminyl radicals, $NR_2^{\bullet}$, or amine radical cations, $NR_3^{\bullet+}$, play an important role in many chemical (*1*–*3*) and biological processes (*4*). Generally, these radicals are highly reactive short-lived intermediates, formed thermally or photolytically by homolytic bond cleavage or by N oxidation. However, the electron deficiency at the nitrogen center can be mitigated by electron-donating R groups, such as O, S, and phenyl, through resonance (Scheme 1, Eq. a) (*5*, *6*). Sterically protected radicals of this type can be isolated (*7*–*9*).

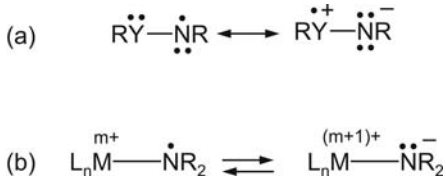
Transition metal–coordinated aminyl radicals are harder to classify. These species have long been sought because of their close resemblance to metal phenoxyl radical complexes, $[ML_n(OR^{\bullet})]$, which are implicated in an important class of metalloenzyme oxidations (*10*, *11*). In contrast to the resonance-stabilized radicals, in the metal complexes the unpaired electron may be more discretely localized, either at N or at the metal: The aminyl (Scheme 1, Eq. b) (left) and amide (right) are distinct electronic states. In some cases, an equilibrium between both states has been observed (redox isomerism) (*12*). The amide form best represents the electronic ground state configuration of most complexes studied so far (*13*, *14*). Recently, persistent anilinyl radical metal complexes were generated in solution but could not be isolated (*15*).

Here we report the synthesis and isolation of a stable dialkylaminyl radical complex **3**, which we characterized by x-ray diffraction and high-resolution electron paramagnetic resonance (EPR) methods. These data, together with density functional theory (DFT) calculations, support electron localization at N and clarify the factors underpinning metal stabilization of aminyls. The compound consists of a $trop_2N^{\bullet}$ radical coordinated to a cationic Rh center (where trop is 5-H-dibenzo[a,d]cycloheptene-5-yl). The Rh also bears a 2,2′-bipyridyl (bipy) ligand and an outer-sphere triflate (OTf^-) counteranion.

We accessed radical **3** by oxidation of the red rhodium amide precursor **2**, (Scheme 2), which in turn was prepared by quantitative deprotonation of amine complex **1** with potassium *tert*-butoxide base. A nearly quantitative yield of orange microcrystalline **1** resulted from reaction of $[Rh(cod)_2]^+OTf^-$ (where cod is cyclooctadiene) with the easily accessible amine $trop_2NH$ (*16*) and bipy in acetonitrile.

The acid dissociation constant (pK_a) value of **1** was estimated by reaction with a reference base according to $\mathbf{1} + ref^- \leftrightarrows \mathbf{2} + refH$ in deuterated dimethylsulfoxide ($DMSO[d_6]$) solvent. The imidazole/imidazolide pair (pK_a = 18.6) (*17*) was used as reference $refH/ref^-$. The equilibrium constant, $K = ([\mathbf{2}] \times [refH])/([\mathbf{1}] \times [ref^-])$, was determined by ^1H nuclear magnetic resonance (NMR) spectroscopy and used in the equation $pK_a(\mathbf{1}) = pK_a(refH) - \log K$. The mean value of several measurements gave $pK_a(\mathbf{1}) = 18.7(2)$. Compared to the typical pK_a of free amines (*18*), this value is roughly 10 orders of magnitude lower, indicating considerable acidification of the amine upon coordination to a Rh(I) center.

In DMSO containing 0.1 M of nBu_4NPF_6 electrolyte, the amide complex **2** is reversibly oxidized to the radical cation complex $[Rh(trop_2N)(bipy)]^{\bullet+}OTf^-$ (**3**) at an electrode potential $E° = -0.55$ V versus the ferrocene/ferrocenium couple [$E°(Fc/Fc^+)$ = 0.0 V]. The Rh amide is much easier to oxidize than are most amines [$NR_3 - e^- \rightarrow NR_3^{\bullet+}$: $E°$(irreversible) ranges from +0.4 to



Scheme 1. Resonance forms of stabilized aminyl radicals. (a) Donor substituted; (b) metal coordinated.

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+1.3 V (versus Fc/Fc^+)]. Chemical oxidation of **2** with ferrocenium triflate in tetrahydrofuran (THF) gives a quantitative yield of **3** as red crystals. From the cycle of H^+ loss (**1** → **2**) and e^- loss (**2** → **3**), we estimated the N-H homolytic bond dissociation energy (BDE; **1** → **3**): A value of 361(2) kJ mol^{-1} was calculated using the equation, BDE = 1.37pK_a + 23.1$E°_{Fc/Fc+}$ + C (C = 303.5 kJ mol^{-1} for DMSO) (*19*).

The solid-state structures of **1**, **2**, and **3** were determined by x-ray diffraction (Fig. 1). The cation in **1** and the rhodium amide **2** have similar structures. The sums of bond angles around the trop nitrogen N_1 [$\Sigma° = 2 \times$ (C-N-Rh) + C-N-C] are 345.1° and 341.5°, respectively. Deprotonation reduces the Rh-N_1 bond length from 2.090(1) Å in **1** to 2.045(3) Å in **2**. Oxidation to radical cation **3** causes a further Rh-N_1 bond shortening to 1.936(3) Å. The coordination sphere around N_1 becomes almost trigonal planar ($\Sigma°$ = 358.9) in **3**. These observations suggest that the oxidation of **2** occurs mainly at the N_1 atom. The slight lengthening of the Rh-C bonds in **3** [average 218.8(4) pm] with respect to **1** [average 216.4(2) pm] and **2** [average 213.9(4) pm] indicates some delocalization of the positive charge onto the metal center. (Details of the synthesis and structure solution are given in the supporting online material.)

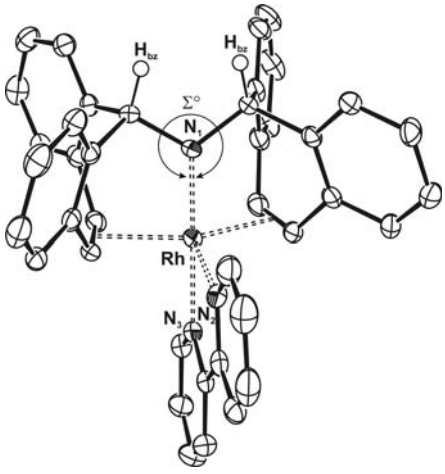
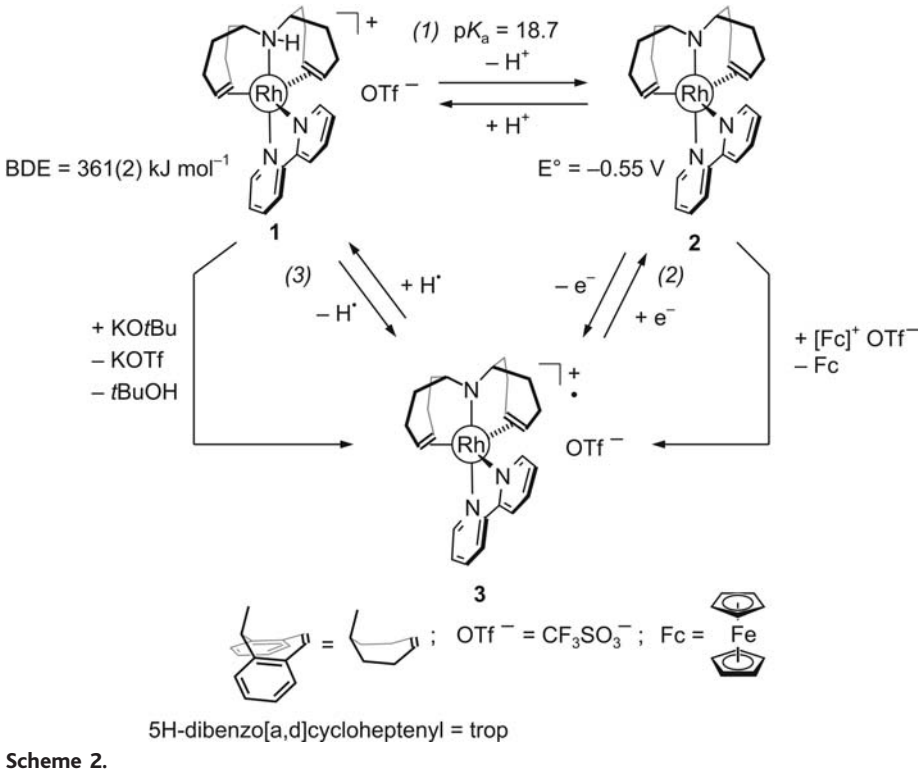


Fig. 1. Structure of **3**. Hydrogen atoms (apart from the two benzylic ones, H_{bz}) and the $CF_3SO_3^-$ anion are omitted.



Scheme 2.

Advanced pulse EPR methods (*20*) were applied to study the electronic structure of **3** in further detail. Our primary goal was to distinguish the aminyl structure from the Rh(II) amide. The continuous wave (CW) EPR spectrum of a solution of pure **3** in a THF-acetone mixture at S-band (2.44 GHz) is well resolved and is shown in Fig. 2A together with the simulation (A_{iso} values in table S1). The echo-detected EPR frozen solution spectrum at Q-band (35.3 GHz) allowed the determination of the principal

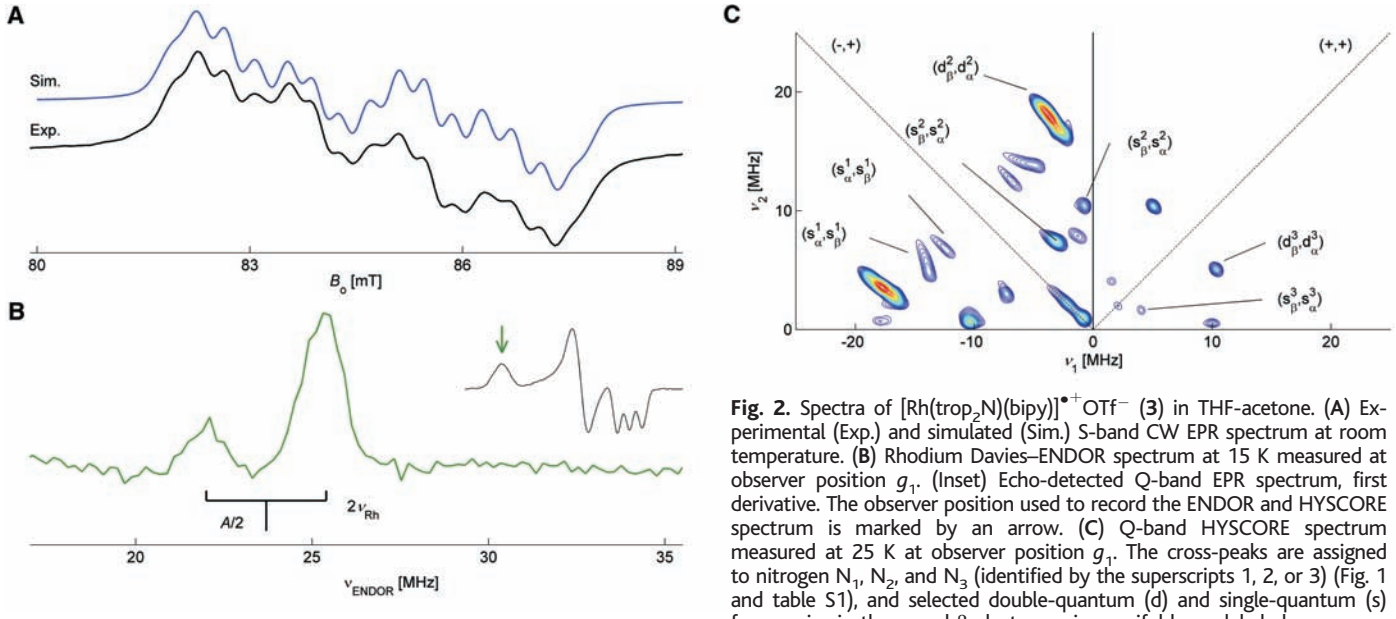


Fig. 2. Spectra of $[Rh(trop_2N)(bipy)]^{\bullet+}OTf^-$ (**3**) in THF-acetone. (**A**) Experimental (Exp.) and simulated (Sim.) S-band CW EPR spectrum at room temperature. (**B**) Rhodium Davies–ENDOR spectrum at 15 K measured at observer position g_1. (Inset) Echo-detected Q-band EPR spectrum, first derivative. The observer position used to record the ENDOR and HYSCORE spectrum is marked by an arrow. (**C**) Q-band HYSCORE spectrum measured at 25 K at observer position g_1. The cross-peaks are assigned to nitrogen N_1, N_2, and N_3 (identified by the superscripts 1, 2, or 3) (Fig. 1 and table S1), and selected double-quantum (d) and single-quantum (s) frequencies in the α and β electron spin manifolds are labeled.

values of the rhombic g matrix [g_1 = 2.0822(2), g_2 = 2.0467(2), g_3 = 2.0247(2)] and the large nitrogen hyperfine coupling of 98 MHz along g_3 (inset in Fig. 2B and fig. S1). The small anisotropy of the g matrix indicates a spin-orbit interaction weaker than the one expected for a Rh(II) complex (*21*).

To obtain a more detailed picture of the spin density distribution, the anisotropic component of the hyperfine interactions is also required. In our frozen-solution CW EPR spectra these interactions are not resolved, but they are obtainable from pulse EPR techniques. Davies-ENDOR (electron-nucleus double resonance) spectra enabled the determination of the hyperfine couplings of the protons in benzyl positions H_{bz} (fig. S2) and of the rhodium hyperfine coupling along g_1 (Fig. 2B). In the HYSCORE (hyperfine sublevel correlation) spectrum (Fig. 2C), cross-peaks from nitrogen nuclei N_1 and N_2 (Fig. 1) are observed predominately in the (−,+)-quadrant because they are strongly coupled ($|A| > 2|\nu_N|$, where ν_N is the nitrogen Larmor frequency). Cross-peaks of the weakly coupled nitrogen nucleus N_3 with $|A| < 2|\nu_N|$ appear predominately in the (+,+)-quadrant. Measurements at several observer positions allowed us to determine the principal values and orientations of the hyperfine and nuclear quadrupole tensors (table S1; figs. S3 and S4).

The large isotropic hyperfine coupling constant of N_1 with A_{iso} = 45.1 ± 0.5 MHz is comparable to those of short-lived free dialkylaminyl radicals [compare $Me_2N^\bullet$ with A_{iso} = 41.4 MHz (*22*)], and the small anisotropy of the g matrix suggests that the unpaired electron is located mainly at the nitrogen center (*23*). The pronounced anisotropy of the hyperfine coupling of N_1 implies that the unpaired electron resides in an orbital with high p-character, in full accordance with the trigonal-planar environment at N_1 determined by x-ray structural analysis. The spin density on N_1 can be calculated from the isotropic and dipolar (T = 26.7 ± 2.0 MHz) part of the hyperfine tensor by comparison to calculated values for the corresponding atomic orbitals (isotropic and dipolar hyperfine couplings for nitrogen are 1443.9 MHz for the 2s orbital and 49.8 MHz for a 2p orbital). This analysis yields a spin density of 57 ± 4% on N_1, of which 3.1 ± 0.1% resides in the 2s orbital and 54 ± 4% in a 2p orbital. Complex **3** is thus best described as an aminyl radical rhodium(I) complex, $[Rh(I)(trop_2N^\bullet)(bipy)]^+OTf^-$, and not as a rhodium(II) amide complex, $[Rh(II)(trop_2N^-)(bipy)]^+OTf^-$.

These conclusions are corroborated by DFT calculations (see supporting online material) performed for the radical cation $[Rh(I)(trop_2N^\bullet)(bipy)]^+$ in **3**. Plots of the spin density distribution and of the singly occupied molecular orbital (SOMO) are displayed in Fig. 3, A and B, respectively. Calculations give a spin density of 56% on N_1 (2% in the 2s-type orbital, 54% in the 2p-type orbital). The corresponding calculated hyperfine couplings are A_{iso} = 30.1 MHz and T = 26.7 MHz. For Rh, a spin density of 30% is calculated. The SOMO is antibonding with respect to the Rh-N_1 bond. In the diamagnetic amide **2**, this orbital is occupied with two electrons, which enforce the antibonding interaction. Thus, the Rh-N_1 bond shortens and strengthens upon oxidation of **2** to **3**. In agreement with a proposal made by Wieghardt and colleagues (*15*), this antibonding interaction is the likely reason for the low oxidation potential of **2**.

Unlike the highly air-sensitive amide **2**, the aminyl radical complex **3** is stable in the solid state for months and in organic solvents at least for days, and its EPR signal persists for hours when the sample is brought into contact with air or when water is added.

A typical reaction of radicals is H-atom abstraction. We tested the reactivity of **3** toward substrates of varying H donor ability (Eq. 1 and Table 1):

$$[Rh(I)(trop_2N^\bullet)(bipy)]^+(\mathbf{3}) + HX \rightarrow [Rh(trop_2NH)(bipy)]^+(\mathbf{1}) + \tfrac{1}{2}\ X-X \quad (1)$$

Radical **3** reacts rapidly and quantitatively with the stannane Bu_3Sn-H and thiophenol (Table 1, entries 1 and 2), which both have lower X-H bond dissociation energies than **1** (BDE = 361 kJ mol^{-1}). The H abstractions from the *tert*-butyl thiol and the biologically relevant thioglycolic acid methyl ester are slightly endothermic and slower (entries 3 and 4), but are driven by the formation of the disulfides, which make the overall process exothermic by ~300 kJ mol^{-1} (*24*).

No reaction is observed with phenol (entry 5), nor with triphenylsilane (entry 6) or the phenyl-substituted methanes (entries 7 and 8), although the latter three reactions are thermodynamically favored.

The kinetic barrier (> 70 kJ mol^{-1}) classifies the aminyl radical complex **3** as a nucleophilic radical within the terminology of radical atom transfer reactions (*25*, *26*): The transition state {[Rh]N(δ^+)•••H•••X(δ^+)} is unfavorably polarized and high in energy with X = •SiR_3 or •CR_3, which are themselves nucleophilic radicals. In contrast, the transition states {[Rh]N(δ^+)•••H•••SR(δ^-)} with complementary electrophilic thiyl radicals •SR are lower in energy.

The acidifying effect of the Rh(I) center on a coordinated NH group (small pK_a) and the strengthening of the Rh-N bond upon formation of the aminyl radical complex **3** allow its formation under very mild conditions. Aminyl radicals, $R_2N^\bullet$, are converted into activated electrophilic radicals, $R_2XN^\bullet$, upon protonation or complexation to Lewis-acids (where X is H^+, $LiBF_4$, $MgBr_2$, or BF_3) (*27*). However, coordination to a soft metal center like Rh(I) has a stabilizing effect and apparently does not change the nucleophilic character of aminyl radicals. It is hoped that this concept will extend the chemistry of heteroatom-centered

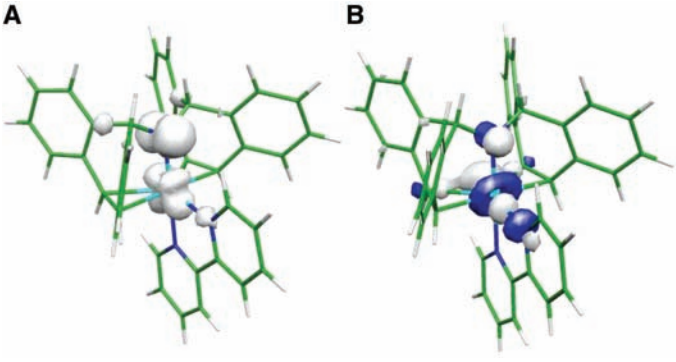


Fig. 3. Plots of the spin density (**A**) and the SOMO (**B**) in $[Rh(I)(trop_2N^\bullet)(bipy)]^+$.

Table 1. Substrates HX used in Eq. 1. The bond dissociation energies (BDEs) are given in kJ mol^{-1}.

Entry	HX	BDE (X-H)	Reaction	Product X-X
1	Bu_3Sn-H	308.5	Yes (fast)	Bu_3Sn-$SnBu_3$
2	PhS-H	348.7	Yes (fast)	PhS-SPh
3	*t*BuS-H	>380.0	Yes (slow)	*t*BuS-StBu
4	$MeOOCCH_2S$-H	>380.0	Yes (slow)	$MeOOCCH_2S$-SCH_2COOMe
5	PhO-H	376.0	No	No
6	Ph_3Si-H	356.0	No	No
7	Ph_2CH-H	343.0	No	No
8	Ph_3C-H	339.0	No	No

radicals and lead to new synthetically useful applications.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/307/5707/235/DC1
Materials and Methods
Figs. S1 to S4
Tables S1 and S2
References

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Encapsulation of Molecular Hydrogen in Fullerene C_{60} by Organic Synthesis

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In spite of their importance in fundamental and applied studies, the preparation of endohedral fullerenes has relied on difficult-to-control physical methods. We report a four-step organic reaction that completely closes a 13-membered ring orifice of an open-cage fullerene. This process can be used to synthesize a fullerene C_{60} encapsulating molecular hydrogen, which can be isolated as a pure product. This molecular surgical method should make possible the preparation of a series of C_{60} fullerenes, encapsulating either small atoms or molecules, that are not accessible by conventional physical methods.

Endohedral fullerenes, the closed-cage carbon molecules that incorporate atoms or a molecule inside the cage (*1*–*6*), are not only of scientific interest but are also expected to be important for their potential use in various fields such as molecular electronics (*7*), magnetic resonance imaging [as a contrast agent (*8*)], and nuclear magnetic resonance (NMR) analysis (*9*, *10*). However, development of their applications has been hampered by a severe limitation in their production, which has relied only on physical methods, such as co-vaporization of carbon and metal atoms (*2*, *3*) and high-pressure/high-temperature treatment with gases (*9*–*14*), that are difficult to control and yield only milligram quantities of pure product after laborious isolation procedures.

An alternative approach to synthesizing endohedral fullerenes is "molecular surgery," in which the cage is opened and then closed in a series of organic reactions (*15*, *16*). For example, an open-cage C_{60} derivative **1** with a 14-membered ring orifice has been synthesized (*17*), and the insertion of molecular hydrogen into **1** in 5% yield has also been achieved (*15*). However, the closure of its orifice was not attempted. A C_{60} derivative **2**, which we synthesized recently (*18*), has a 13-membered ring orifice with a sulfur atom on its rim and a relatively circular shape compared with the elliptic orifice of **1**. This opening has enabled us to insert molecular hydrogen through this orifice in 100% yield (*19*). When matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was conducted under enhanced laser power on compound **2** encapsulating hydrogen (H_2@**2**), we observed a molecular ion peak for $H_2@C_{60}$ at a mass-to-charge ratio (*m/z*) of 722 (*19*). This result suggested that H_2@**2** could be a precursor for $H_2@C_{60}$ in an actual chemical transformation. We now report the synthesis of 100% pure $H_2@C_{60}$ from H_2@**2** (Scheme 1).

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Encapsulated H_2 escapes from the cage when the compound H_2@**2** is heated above 160°C (*19*), so high temperatures must be avoided if the chemical synthesis of $H_2@C_{60}$ is attempted from H_2@**2**. With such a precaution being taken, we performed a stepwise reduction of the orifice size of H_2@**2** and completed its closure by a thermal reaction. The application of heat to the last step did not cause a serious loss of H_2, because the orifice size was already reduced sufficiently to prevent such loss.

The first step involved the oxidation of the sulfide unit (-S-) in H_2@**2** to a sulfoxide unit (>S=O) to give H_2@**3**. The resulting >S=O unit was removed by a photochemical reaction to produce H_2@**4** (Fig. 1, steps A and B) (*20*). Both reactions proceeded at room temperature with yields of 99% and 42% (68% for step B based on consumed H_2@**3**), respectively. The MALDI-TOF mass spectrum of H_2@**4** exhibited the molecular ion peak of $H_2@C_{60}$ as a base peak, indicating its enhanced accessibility from H_2@**4** as compared to H_2@**2**. The spectrum, however, also showed the presence of empty C_{60} in 20% yield relative to $H_2@C_{60}$ and indicated that further reduction of the orifice size was needed. Thus, in the next step, two carbonyl groups in H_2@**4** were reductively coupled by the use of Ti(0) (*21*) at 80°C, to give H_2@**5** with an eight-membered ring orifice (Fig. 1, step C).

At each process in these three steps, complete retention of encapsulated H_2 was confirmed by observing the characteristically upfield-shifted NMR signal of the incorporated H_2. The integrated signal intensity exactly corresponded to 2.00 ± 0.05 H for the signals at a chemical shift δ of –6.18 parts per million (ppm) in H_2@**3**, at –5.69 ppm in H_2@**4**, and at –2.93 ppm in H_2@**5**, with reference to the 2.00 H signal for two aromatic protons. The gradual downfield shift of the hydrogen signal

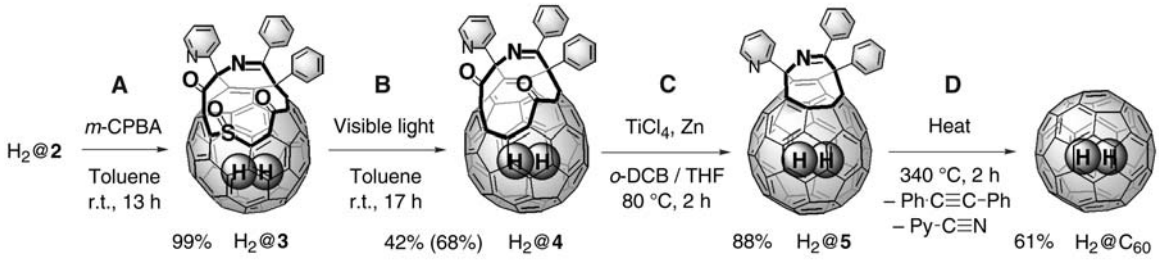


Fig. 1. Size reduction and closure of the orifice of the open-cage fullerene encapsulating hydrogen, in a four-step process. Percentage values are product yields; that shown in parenthesis is that based on the consumed precursor. *m*-CPBA, r.t., and *o*-DCB stand for *m*-chloroperbenzoic acid, room temperature, and *o*-dichlorobenzene, respectively.

observed at steps B and C reflects the formation at each step, within the fullerene cage, of a fully π-conjugated pentagon, which exerts a strong deshielding effect through its paramagnetic ring currents (*22*).

Finally, complete closure of the orifice was achieved by heating powdery H_2@**5** in a glass tube at 340°C for 2 hours under vacuum (Fig. 1, step D). The desired product $H_2@C_{60}$ (118 mg, contaminated with 9% empty C_{60}) was obtained in 67% yield by passing a carbon disulfide solution of the crude product through a silica-gel column. Similar results were obtained when H_2@**5** was heated at 300°C for 24 hours, at 320°C for 8 hours, or at 400°C for 2 min. Thus, $H_2@C_{60}$ was synthesized in a total yield of 22% from H_2@**2**, which can be obtained in 40% yield from consumed C_{60} (*18*, *19*).

We presume that the closure of the orifice takes place by way of a thermally allowed [π2s + π2s + π2s] electrocyclization reaction that produces two cyclopropane rings (Fig. 2). Sequential radical cleavage and a retro [σ2s + σ2s + σ2s] reaction produce C_{60} by splitting off 2-cyanopyridine and diphenylacetylene.

The ^{13}C NMR spectrum of the desired product exhibited a signal at δ = 142.844 ppm together with a very small signal at δ = 142.766 ppm (Fig. 3A), the latter corresponding exactly to the signal of empty C_{60}. In an expanded spectrum obtained with 56,576 data points for a 50-ppm spectral width, the integrated peak areas of these signals yield an estimated ratio of $H_2@C_{60}$ and empty C_{60} of 10:1.

We separated $H_2@C_{60}$ from C_{60} through recycling high-performance liquid chromatography on a semipreparative Cosmosil Buckyprep column (two directly connected columns, 25 cm by 10 mm inner diameter, with toluene as a mobile phase; flow rate, 4 ml min^{-1}; retention time, 395 min for C_{60} and 399 min for $H_2@C_{60}$). Isolated $H_2@C_{60}$ was judged to be 100% pure on the basis of a single ^{13}C NMR signal at 142.844 ppm (Fig. 3B), the results of high-resolution fast-atom-bombardment mass spectrometry (calculated molecular weight for $C_{60}H_2$: 722.0157; found: 722.0163), and the agreement of the

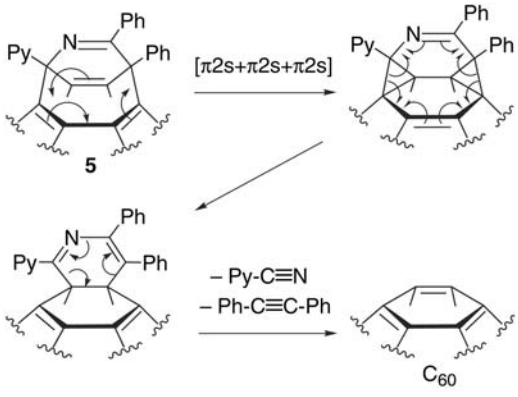


Fig. 2. Proposed reaction mechanism for the formation of C_{60} from compound **5** by heating. Only the tops of the molecules are shown. Ph and Py stand for phenyl and 2-pyridyl groups, respectively.

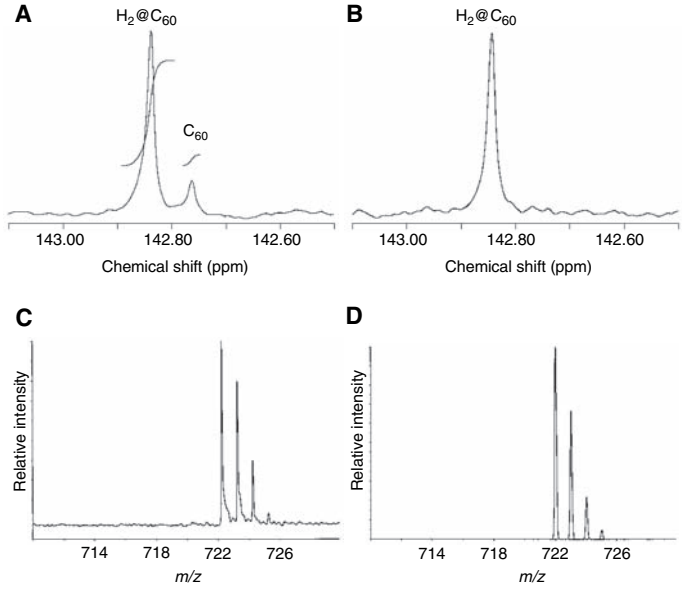
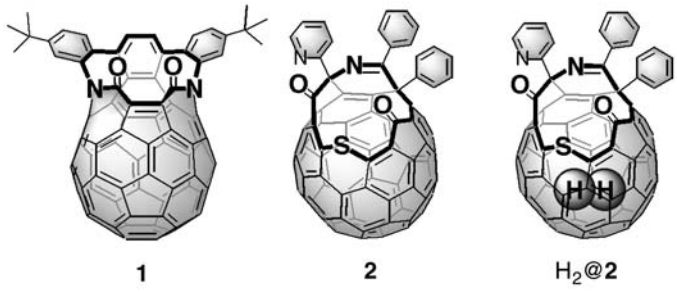


Fig. 3. Structural characterization of $H_2@C_{60}$. (**A**) Expanded ^{13}C NMR spectrum (75 MHz, *o*-DCB-d_4) of $H_2@C_{60}$ contaminated by 9% C_{60}. (**B**) Expanded ^{13}C NMR spectrum (75 MHz, *o*-DCB-d_4) of purified $H_2@C_{60}$. (**C**) MALDI-TOF mass spectrum (positive ionization mode, dithranol matrix) of purified $H_2@C_{60}$. (**D**) Predicted isotope distribution pattern for $H_2@C_{60}$.

Scheme 1.



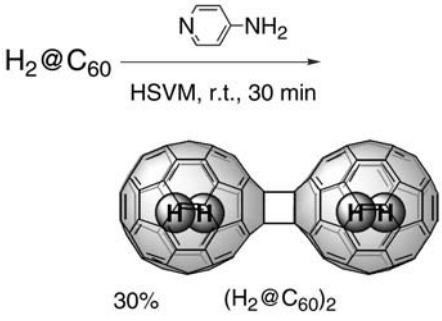


Fig. 4. Mechanochemical solid-state dimerization of $H_2@C_{60}$ by the use of a high-speed vibration milling (HSVM) technique.

observed and predicted isotope distribution patterns in the MALDI-TOF mass spectrum (Fig. 3, C and D), in addition to correct elemental analysis for hydrogen (calculated for $C_{60}H_2$: C, 99.72, and H, 0.28%; found: C, 99.04, and H, 0.24%).

The very small downfield shift (0.078 ppm) observed for the ^{13}C NMR signal of $H_2@C_{60}$ (as compared to empty C_{60}) indicates that the electronic property of the fullerene cage is largely unaffected by the encapsulation of H_2. The ultraviolet-visible and infrared spectra of $H_2@C_{60}$ are also exactly the same as those of empty C_{60}. This situation contrasts with the cases of $Kr@C_{60}$ (*13*) and $Xe@C_{60}$ (*12*), in which larger downfield shifts are observed (0.39 ppm and 0.95 ppm, respectively), caused by appreciable electronic and van der Waals interactions between the C_{60} cage and the encapsulated atoms, which are much larger than H_2.

The ^1H NMR signal for the encapsulated hydrogen of $H_2@C_{60}$ in *o*-dichlorobenzene-d_4 was observed at $\delta = -1.44$ ppm, which is 5.98 ppm upfield-shifted relative to the signal of dissolved free hydrogen. The extent of this upfield shift is comparable to that observed for $^3He@C_{60}$ (6.36 ppm) (*9*, *10*) in ^3He NMR relative to free ^3He. This result shows that the shielding effect of total ring currents of the C_{60} cage is nearly the same, regardless of the paramagnetic species inside the cage.

The irrelevance of the encapsulated H_2 to the electronic character of the outer cage was also demonstrated by cyclic voltammetry (0.5 mM in *o*-dichlorobenzene with 0.05 M Bu_4NBF_4 for reduction and 0.5 mM in 1,1,2,2-tetrachloroethane with 0.1 M Bu_4NPF_6 for oxidation). The voltammogram of $H_2@C_{60}$ exhibited four reversible reduction waves and one irreversible oxidation peak at the same potentials as C_{60}, within an experimental error of ±0.01 V.

In order to clarify the reactivity of $H_2@C_{60}$, the solid-state mechanochemical [2+2] dimerization reaction (*23*) was conducted. A mixture of $H_2@C_{60}$ and 1 molar equivalent of 4-aminopyridine as the catalyst (*24*) was vigorously shaken by the use of a high-speed vibration mill for 30 min under N_2 according to our previous procedure (*23*, *24*). The ^1H NMR spectrum of the product mixture exhibited a signal at $\delta = -4.04$ ppm of the [2+2] dimer, $(H_2@C_{60})_2$, and a signal of unchanged $H_2@C_{60}$ at $\delta = -1.44$ ppm, in an integrated ratio of 3:7. This result indicates that the dumbbell-shaped dimer of $H_2@C_{60}$ is formed in the same yield as that for the reaction of empty C_{60} (*24*) (Fig. 4). No effect of the encapsulated H_2 was observed upon reactivity of the C_{60} cage. The extent of the upfield shift of the ^1H NMR signal (2.60 ppm) observed for the dimer $(H_2@C_{60})_2$ was similar to that observed upon the same dimerization reaction in ^3He NMR (2.52 ppm) (*24*) for ^3He encapsulated in the ratio of ~0.1% in C_{60} (*9*, *10*).

The endohedral fullerene $H_2@C_{60}$ is nearly as stable as C_{60} itself. For example, the encapsulated H_2 does not escape even when heated at 500°C for 10 min. Thus, $H_2@C_{60}$ can be viewed as a stable hydrocarbon molecule that has neither C-H covalent bonds nor C•••H interactions. It is likely that our method could be used to synthesize endohedral fullerenes such as $D_2@C_{60}$ and $HD@C_{60}$, as well as the homologous series with C_{70}. Our work here complements the total chemical synthesis of C_{60} recently achieved by Scott and co-workers (*25*) and implies that organic synthesis can be a powerful means for the production of yet unknown classes of endohedral fullerenes.

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www.sciencemag.org/cgi/content/full/307/5707/238/DC1
Materials and Methods
Figs. S1 to S9
References

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Corrected Late Triassic Latitudes for Continents Adjacent to the North Atlantic

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We use a method based on a statistical geomagnetic field model to recognize and correct for inclination error in sedimentary rocks from early Mesozoic rift basins in North America, Greenland, and Europe. The congruence of the corrected sedimentary results and independent data from igneous rocks on a regional scale indicates that a geocentric axial dipole field operated in the Late Triassic. The corrected paleolatitudes indicate a faster poleward drift of ~0.6 degrees per million years for this part of Pangea and suggest that the equatorial humid belt in the Late Triassic was about as wide as it is today.

Paleomagnetism is used to determine ancient latitude, but its reliability depends on two assumptions: (i) that the time-averaged geomagnetic field is closely approximated by that of a geocentric axial dipole (GAD), and (ii) that there is no systematic bias in how the geomagnetic field is imprinted in rocks. Although the GAD hypothesis (*1*) is supported by paleomagnetic data for the past few million years (*2*, *3*), departures from the GAD model

have been invoked to explain anomalously shallow directions observed in some older rocks (*4*–*6*). On the other hand, a shallow bias or inclination error (*7*) has been found in laboratory redeposition experiments and in some modern natural sediments (*8*). A good example of the ambiguity in distinguishing between a non-GAD field and recorder bias is found in the paleomagnetic record from continental basins that developed during rifting of the Pangea supercontinent in the early Mesozoic and are now distributed along the margins of the North Atlantic (Fig. 1). One of the largest and best studied of the rift basins is the Newark basin in eastern North America, where more than 5000 m of strata (mainly continental redbeds) were recovered in scientific drilling (*9*) and which yielded a 35 million year (My)–long record of latitudinal drift calibrated by an astronomically tuned geomagnetic polarity time scale (*10*, *11*). The average paleomagnetic inclinations from the Dan River basin (*12*) and the Fundy basin (*13*) indicate low paleolatitudes that are consistent with the Newark basin data. However, paleomagnetic directions from the more distant Jameson Land basin in Greenland (Fig. 1) are anomalously shallow and imply a paleolatitude ~10° lower than predicted from coeval sections in North America (*14*). This discrepancy is too large to be explained by uncertainties in the reconstruction of Greenland to North America (*15*, *16*). Therefore, either the magnetizations of the sedimentary rocks are biased by inclination error or the Late Triassic time-averaged field included large nondipole (axial octupole) contributions (*4*).

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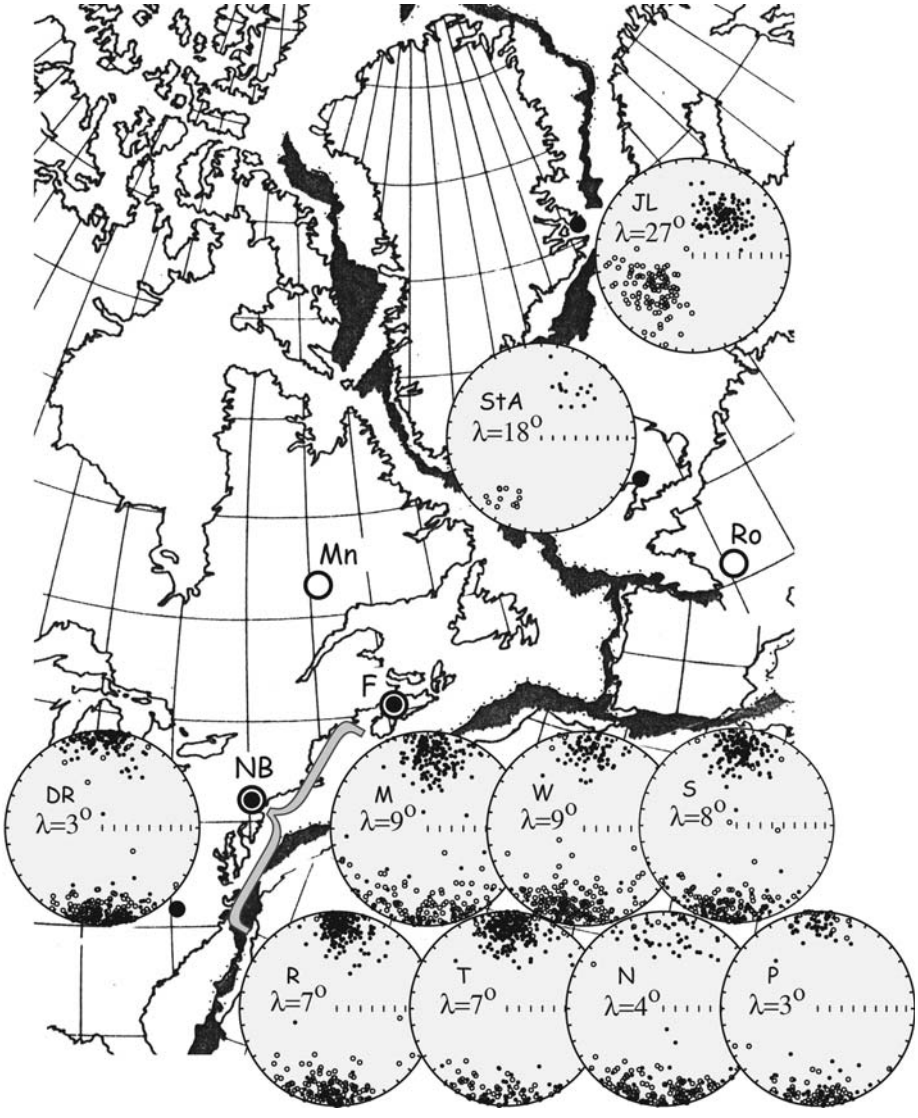

Fig. 1. Paleomagnetic sampling localities of key Late Triassic and earliest Jurassic sedimentary and igneous rock units plotted on a Pangea reconstruction (*16*). For reference, present-day latitude/longitude grids (5° by 5°) are shown for each continent. Insets give the paleomagnetic direction data (solid and open symbols are on the lower and upper hemisphere, respectively, of equal-area projections) and the implied paleolatitudes (λ) from each section: DR, Dan River basin (*12*); NB, Newark basin with data from seven drill cores labeled with the first initial of the drill core (M, W, S, R, T, N, and P) (Table 1) (*10*); JL, Jameson Land basin (*14*); StA, St. Audrie's Bay section (*19*). Other data discussed in the text are from F, the Fundy basin (*13*, *22*); Mn, the Manicouagan impact structure (*24*, *25*); and Ro, the Rochechouart impact structure (*23*). The data are summarized in Table 1.

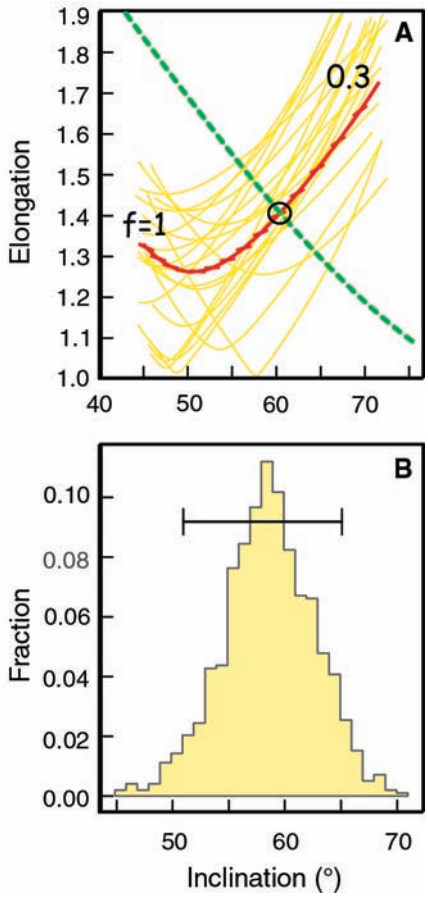

Fig. 2. E/I analysis of sample characteristic magnetization directions from Fleming Fjord Formation, Jameson Land, Greenland (*12*). (**A**) The trajectory (solid red line) of mean inclination versus elongation calculated for the Jameson Land data shown in the inset to Fig. 1, as the data are inverted with values for *f* ranging from 0.3 to 1.0. Yellow lines are examples of bootstrapped trajectories. The predicted E/I trend with latitude of the TK03.GAD model (*17*) is shown as the blue dashed line; the E/I of the data consistent with the model is circled. (**B**) A histogram of 1000 bootstrapped intersections of the kind shown in (A) from bootstrapped curves. The 95% confidence bounds on the corrected inclination are also shown (Table 1).

To address this problem, we employed a method to recognize and correct for inclination error that is based on a statistical geomagnetic field model (TK03.GAD) (*17*) consistent with global paleomagnetic data from lavas for the past 5 My. The model treats the geomagnetic field as the sum of the axial dipole component with a nonzero mean plus the nonaxial dipole contributions and higher order terms with means of zero. Each of the terms is drawn from normal distributions, the variance of which depends in a simple way on degree and order. This is similar to Constable and Parker's Giant Gaussian approach to paleosecular variation modeling (*18*). Like all GAD models, TK03.GAD predicts that the mean field inclination, I, is a function of latitude, λ:

$$\tan I = 2 \tan \lambda \quad (1)$$

The model also predicts that secular variation will result in a distribution of virtual geomagnetic poles that is essentially circular at any observation site. This implies that the distribution of directions (declination and inclination) will be increasingly elongate as the observation site latitude decreases from the pole(s) to the equator. Accordingly, the model predicts a unique elongation/inclination (E/I) relationship for directional distributions. If the directions were affected by inclination error (either during deposition or imparted by compaction), the observed inclination, I_o, will be related to the ambient field inclination, I_f, by

$$\tan I_o = f \tan I_f \quad (2)$$

where f is the flattening factor (*7*). Inclination error affects the distribution of directions by increasing the east-west elongation while decreasing inclination. A nonzero axial octupole contribution of the same sign as the axial dipole will also decrease inclination, but it will increase elongation in the up-down plane. Hence, the two mechanisms, each capable of producing shallow inclinations, have different effects on the distribution of directions. If inclination error is the cause of the shallow bias, the data can be inverted, with the inverse of Eq. 2 searching for a value of f that yields an elongation and inclination combination consistent with the field model; the corrected mean inclination should provide a more accurate estimate of latitude according to Eq. 1. The hypothesis that the statistical properties of the geomagnetic field in remote epochs were similar to those of the more recent [0 to 5 My ago (Ma)] geomagnetic field can be tested for the Late Triassic by the ability of the E/I method to produce an internally consistent latitudinal framework.

We analyzed characteristic directions from Jameson Land that were isolated by progressive thermal demagnetization in 222 samples, which include data for 150 samples reported previously (*14*) and 72 additional samples collected in a subsequent field season (Fig. 1 and Table 1). The overall mean direction (after small bedding tilt corrections and inverting to common polarity) was a declination (D) of 43.1°, with I of 45.1°, similar to the original subset of 150 samples (D = 43.6°, I = 45.3°). The distribution of directions is elongated east-west, which points to inclination error in producing the anomalously shallow mean inclination (Fig. 2). The data set was inverted with values of f from 0.3 to 1.0. For each value of f, we calculated the elongation and mean inclination of the distribution (*17*). The evolution of E/I with f (Fig. 2A) crosses the curve predicted by the TK03.GAD model at I = 60° for f = 0.58. Bootstrap resampling of the data yielded 1000 crossings (Fig. 2B) and 95% confidence bounds of 51 to 65°. Therefore, results of the E/I analysis indicate a direction that is ~15° steeper than the initial mean inclination (Table 1). Results of E/I analysis on data from the Dan River basin (*12*) and from the seven drill cores from the Newark basin (*10*) (fig. S1) give distributions (each based on 148 to 336 samples) that are elongated east-west, consistent with inclination error; best-fit f values range from 0.40 to 0.66 (Table 1).

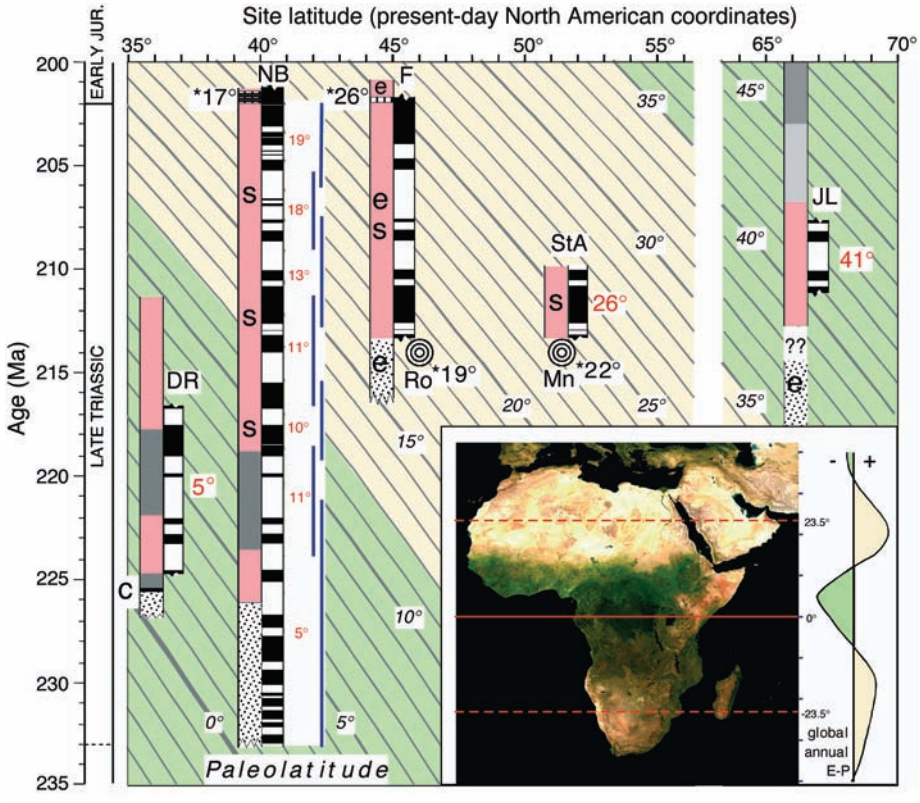

Fig. 3. Paleolatitude nomogram for the Late Triassic and earliest Jurassic of a portion of Pangea. Paleolatitude contours are based on a smoothed progression of latitudes that are calculated from corrected mean inclinations, according to the E/I method, from seven drill cores from the Newark basin (NB, with corrected paleolatitudes adjacent to solid lines indicating the age ranges of the cores) and take into account present geographic relationships. Mean paleomagnetic declinations for the Late Triassic of eastern America are typically within a few degrees of north–south (Table 1), and hence differences in present-day latitudes of the sites in North American coordinates closely approximate differences in paleolatitude. Site latitudes for the Jameson Land (JL) section in Greenland (*14*), the St. Audrie's Bay (StA) section (*19*), and the 214-My-old (*27*) Rochechouart impact structure (Ro) (*23*) in Europe were transferred to North American coordinates according to reconstruction parameters from Bullard *et al*. (*16*) (Table 1). The geomagnetic polarity time scale from the NB cores (*11*) was used as the basis of magnetostratigraphic correlation (solid and open bars denote normal and reverse polarity, respectively) and age control. Mean paleolatitudes from corrected inclinations are indicated for JL, StA, and the Dan River basin (DR) (Table 1). Paleolatitudes with asterisks are for igneous rocks from earliest Jurassic (~200 Ma) Central Atlantic Magmatic Province lavas in the NB (*21*) and Fundy (F) basins (*22*) and from the 214-My-old (*26*) Manicouagan (Mn) impact structure in Quebec (*24*, *25*) and Ro. Climate lithofacies are shown for the DR, NB, and F (*35*) and JL (*37*) sections where c is coal, s is saline minerals, and e is eolian deposits; light to dark shading in lithology columns ranges from fine-grained redbeds to black shales, with stippling indicating sandstones. The inset is (left) a Landsat image of Africa (*44*) and (right) the global zonal mean profile of evaporation minus precipitation (E-P) (*45*). Green and yellow colors indicate more humid and arid conditions, respectively, the present-day latitudinal ranges of which are shown on the paleolatitude nomogram for comparison.

Although the data set from St. Audrie's Bay in Somerset, United Kingdom, is rather small (27 sites), the results are included because they are possibly the first for the Late Triassic of Europe to be supported by modern demagnetization techniques and the site-mean directions are available (*19*).

We compared the corrected results, which differ in age as well as location, by constructing a paleolatitude versus age nomogram (*13*) by fitting a second-order polynomial curve to the corrected paleolatitude versus age progression determined from the Newark basin cores (Table 1). We then used that relationship to predict paleolatitudes over the same 35-My interval for tectonically contiguous or reconstructed areas. The corrections for inclination error bring the paleolatitude data for the Triassic basins into agreement (Fig. 3). In particular, the corrected paleolatitude for the previously discrepant result from Jameson Land is now consistent with the paleolatitude predicted from the corrected Newark basin data. The data set from St. Audrie's Bay also falls into line after correction.

As an independent check on the validity of the corrections for inclination error, we can use paleomagnetic data from approximately coeval igneous rock units, which are not subject to inclination error. Lava flows of the Central Atlantic Magmatic Province of the earliest (~200 Ma) Jurassic age (*20*) occur in several of the rift basins in eastern North America. For comparison with the Newark basin sedimentary results, we used a compilation of the most reliable data as determined by Prevot and McWilliams (*21*) for the three major extrusive units in the Newark and nearby Hartford basins. These lava flow data indicate a paleolatitude of 17 ± 5° for the Newark basin (Table 1), which is in much better agreement with the paleolatitude after correction for inclination error (19°) than without correction (~9°), as determined from the immediately underlying (Upper Triassic and lowermost Jurassic) sedimentary rocks of the Passaic Formation in the Newark basin Martinsville core (Fig. 3 and Table 1). Paleomagnetic results from the Upper Triassic and lowermost Jurassic Blomidon Formation in the Fundy basin (*13*) give an anomalously low paleolatitude (~12°), but these inclination-only sedimentary data could not be tested or corrected with the E/I method. Nevertheless, paleomagnetic data (*22*) for the overlying North Mountain Basalt indicate a paleolatitude of 26°, which is consistent with the paleolatitude predicted from the corrected Newark data (Fig. 3). Paleolatitudes from two older igneous rock units also agree with the paleolatitude–age matrix predicted from the corrected Newark basin data (Fig. 3): Rochechouart (*23*) in France and Manicouagan (*24*, *25*) in Quebec, which are impact structures dated at ~214 Ma (*26*, *27*).

With the caveat that these igneous rocks may not adequately average secular variation because of the paucity of cooling units, the overall agreement of the igneous data with the predictions based on the E/I method supports the hypothesis that inclination error pervasively affects the sedimentary rocks we studied. An early study that found no substantial difference in paleomagnetic directions, all of normal polarity, from sediments and igneous rocks in the Newark basin (*28*) was before routine use of progressive thermal demagnetization, which has revealed that a depositional component of normal and reverse polarity with shallow directions in these redbeds is typically masked by a steeper normal polarity thermochemical overprint (*29*). On the other hand, an early-noticed (*30*) discordance in presumed Triassic latitudes between North America and Europe in a Pangea configuration is likely to be an artifact of age differences and inclination error, because it was largely based on a comparison between data from Jurassic igneous rock units in eastern North America and Triassic sedimentary rocks in western Europe. The corrected St. Audrie's Bay data show that Late Triassic latitudes of Europe are consistent with those of North America in a Pangea (*16*) fit.

Slow poleward motion of ~0.3° per My for North America was inferred from the original Newark basin paleomagnetic data (*10*), but these are biased by inclination error, which underestimates paleolatitudinal change. The corrected inclinations indicate a much faster rate of poleward motion of ~0.6° per My from ~235 to 200 Ma, which emphasizes the need for precise spatiotemporal registry of climate-sensitive lithofacies in paleoclimate studies. The overall distribution of such facies within the cyclic successions suggests that generally moist conditions extended from the coal-bearing and black shale units in the Dan River basin (*31*) near the paleoequator and black shales of the Lockatong Formation in the Newark basin at 5 to 10° to where eolian deposits in the Fundy basin (*32*) are encountered at ~15° paleolatitude. The width of the equa-

Table 1. Summary of paleomagnetic data from Late Triassic and earliest Jurassic rocks. Slat and Slon are the latitude and longitude of the sampling localities; entries in parentheses are site locations in Greenland and Europe transferred to North American coordinates according to Bullard *et al.* (*16*). Age is the mean age of the sampled rocks; *n* is the number of data included in the mean values; *D* and *I* are the mean declination and mean inclination of the characteristic magnetization data; λ is paleolatitude calculated from the mean inclination; *f* is the flattening factor determined from E/I analysis, *I*′ is the corrected mean inclination and ±*I*′ is its 95% confidence interval; λ′ is the corresponding corrected paleolatitude and ±λ′ its 95% confidence interval; and Ref. is the literature source for the age and paleomagnetic data.

Locality	Slat (°N)	Slon (°E)	Age (Ma)	*n*	*D* (°)	*I*(°)	λ (°N)	*f*	*I*′ (°)	±*I*′ (°)	λ′ (°N)	±λ′(°N)	Ref.
Sedimentary													
Dan River	36.5	–79.5	221	333	0.5	5.9	3.0	0.59	10	8–12	5.0	4.0–6.1	(*12*)
Newark basin													
Princeton	40.4	–74.6	227	148	0.1	5.2	2.6	0.56	9	5–13	4.5	2.5–6.6	(*10*)
Nursery	40.3	–74.8	221	194	1.8	8.8	4.4	0.40	21	16–25	10.9	8.2–13.1	″
Titusville	40.3	–74.9	217	308	2.9	13.0	6.6	0.63	20	17–23	10.3	8.7–12.0	″
Rutgers	40.5	–74.4	214	336	4.3	14.2	7.2	0.66	21	19–24	10.9	9.8–12.6	″
Somerset	40.5	–74.6	211	309	4.9	15.7	8.0	0.63	24	21–28	12.6	10.9–14.9	″
Weston	40.2	–74.6	207	246	7.4	17.5	8.9	0.49	33	28–37	18.0	14.9–20.6	″
Martinsville	40.6	–74.6	204	302	3.8	18.2	9.3	0.49	34	29–38	18.6	15.5–21.3	″
St. Audrie's Bay	51.2 (51.9)	–3.3 (–40.5)	212	27	31.6	33.4	18.2	0.66	44	33–48	25.8	18.0–29.0	(*19*)
Jameson Land	71.5 (66.2)	–22.7 (–35.3)	209	222	43.1	45.1	26.6	0.58	60	51–65	40.9	31.7–47.0	(*14*)
Igneous													
Newark	40.5	–74.5	201	3	3.5	31.4	17.0 ± 5.0	—	—	—	—	—	(*21*)
North Mountain	45.0	–65.0	201	9	17.7	44.5	26.2 ± 8.6	—	—	—	—	—	(*22*)
Manicouagan	51.4	–68.6	214	11	11.8	38.3	21.6 ± 5.8	—	—	—	—	—	(*24–26*)
Rochechouart	45.8 (46.5)	+0.8 (–36.5)	214	33	226.4	–34.8	19.2 ± 2.6	—	—	—	—	—	(*23*, *27*)

torial humid belt in the Late Triassic was comparable to today's (Fig. 3), a conclusion that contrasts with some previous suggestions of a much more restricted or even dry equatorial belt in the Triassic (*33*, *34*). Poleward motion can explain the generally drier northward and up-section facies pattern in the Mesozoic rift basins of eastern North America (*32*, *35*) as this part of Pangea drifted out of the equatorial humid belt. At the same time, the up-section progression to more humid facies in the Fleming Fjord Formation (*36*, *37*) and the overlying plant-bearing Kap Stewart Formation of latest Triassic and earliest Jurassic age (*38*) in the Jameson Land basin would reflect the drift of this area into the temperate humid belt.

We conclude that the congruence of the corrected paleomagnetic data from sedimentary rocks and independent data from igneous rocks ranging over thousands of kilometers and tens of millions of years indicates that a GAD field similar to that of the past 5 My was operative at least 200 Ma in the Late Triassic and earliest Jurassic. In particular, we see no evidence for a major octupole contribution in either the shapes of the distributions of directions in the sedimentary units or in the geographic distribution of site paleolatitudes. As indicated by other recent studies (*17*, *39–41*), there is thus little empirical basis to invoke persistent departures from the GAD field, especially zonal octupole contributions, to address tectonic problems (*4*, *42*, *43*). Instead, our results suggest that inclination error in sedimentary rocks may be more prevalent than has been supposed, perhaps especially in cases where the magnetizations that have been isolated are most likely to represent a depositional remanence carried by hematite. The success of the E/I method (*17*) to determine the degree of flattening and to correct any bias in inclinations from the distribution of directions should provide motivation for more intensive sampling of sedimentary rock units and for making detailed data more accessible.

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An Astronomical 2175 Å Feature in Interplanetary Dust Particles

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The 2175 angstrom extinction feature is the strongest (visible-ultraviolet) spectral signature of dust in the interstellar medium. Forty years after its discovery, the origin of the feature and the nature of the carrier(s) remain controversial. Using a transmission electron microscope, we detected a 5.7–electron volt (2175 angstrom) feature in interstellar grains embedded within interplanetary dust particles (IDPs). The carriers are organic carbon and amorphous silicates that are abundant in IDPs and in the interstellar medium. These multiple carriers may explain the enigmatic invariant central wavelength and variable bandwidth of the astronomical 2175 angstrom feature.

Much of what is known about grains in space comes from spectral features observed in emission, polarization, and absorption (*1–7*). The 2175 Å peak is by far the strongest feature observed in the ultraviolet (UV)–visible wavelength range along most lines of sight for which it can be measured (Fig. 1, A and B) (*4–7*). The feature is enigmatic: Its central wavelength is almost invariant, but its bandwidth varies from one line of sight to another, suggesting multiple carriers or a single carrier with variable properties. From interstellar abundances of the elements and typical UV transition strengths, the carrier is either oxygen-rich (e.g., oxides or silicates) or carbon-rich (e.g., graphite or organic compounds) (*1–4*, *8*). We searched UV spectra of chondritic IDPs for an extinction feature near the ~2175 Å interstellar feature (Fig. 1). Materials similar to the two most abundant grain types seen in the

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interstellar medium (ISM), amorphous silicates and carbonaceous matter, are found in IDPs (Fig. 2) (*9*). The amorphous silicates are glass with embedded metal and sulfides (GEMS), some with nonsolar O isotopic compositions (*10*–*13*). The carbonaceous matter is a mixture of inorganic and organic carbon, and some of the organic materials exhibit nonsolar D/H, $^{15}N/^{14}N$, and $^{13}C/^{12}C$ ratios comparable in magnitude to those observed in interstellar molecular clouds (*13*–*15*). The nonsolar isotopic signatures establish that these GEMS and carbonaceous subgrains are of interstellar origin.

We used a new-generation transmission electron microscope (TEM) equipped with a monochromator and high-resolution electron energy-loss spectrometer to measure UV spectral properties of portions of IDPs and standards (*16*, *17*). The 0- to ~100-eV region of an energy-loss spectrum, known as the valence electron energy-loss spectroscopy (VEELS) region (*17*), includes the 2175 Å (5.7 eV) UV spectral feature. We used VEELS because the submicrometer dimensions of the subgrains preclude measurement by conventional photoabsorption spectroscopy (PAS). The VEELS data were acquired under conditions in which the positions of VEELS and PAS features are comparable, but PAS typically has ~10 times better wavelength/energy resolution (*18*). A synchrotron light source was used to measure infrared (IR) spectral properties (*17*), and two NanoSIMS (secondary ion mass spectrometry) microprobes were used to measure the isotopic compositions of grains within the same IDPs (*17*).

A VEELS spectrum from the mineral talc ($Mg_3Si_4O_{10}[OH]_2$) shows a peak position and bandwidth that match the photoabsorption feature of hydroxylated amorphous Mg_2SiO_4 (Fig. 1, C and D) (*8*), as well as the astronomical UV feature (Fig. 1, A, B, and D). VEELS spectra from carbonaceous grains in three IDPs exhibit a 5.7-eV feature with average bandwidth (full width at half maximum, FWHM) of 2.6 eV (2.2 μm^{-1}) (Fig. 3, A to C). With increasing O/C ratio, the strength of the 5.7-eV feature increases and the peak of the volume plasmon (the broad peak between 10 and 28 eV) decreases in energy. Energy-loss C and O core scattering edges from the most O-rich regions exhibit a fine structure consistent with carbonyl (or hydroxyl) functional groups (*19*), and IR spectra there exhibit prominent C-H stretch and C=O features at ~3.4 μm and ~5.9 μm, respectively (Fig. 4). Although the signal-to-noise ratio is marginal, because the IR spectrum was acquired from a ~9-μm^2 area ~0.1 μm thick, the overall structure of the C-H stretch feature between 2850 and 3100 cm^{-1} in L2036 G16 (and in other IDPs) is consistent with aliphatic groups bound to other molecules like polycyclic aromatic hydrocarbons (PAHs) (*19*–*21*). 1-Pyrene carboxaldehyde ($C_{17}H_{10}O$) exhibits the ~5.7-eV feature but pyrene ($C_{16}H_{10}$), with no carbonyl group, produces a feature that is shifted to higher energy (~6.1 eV) (Fig. 3, D and E). GEMS produce a 5.7-eV feature with an average bandwidth (FWHM) of 2.9 eV (2.5 μm^{-1}), and the feature strength correlates with hydroxyl (OH^-) content (Fig. 3, F to J). Thus, both organic compounds and amorphous silicates in IDPs may be carriers of a 5.7-eV feature.

The central wavelength of the IDPs' 5.7-eV VEELS feature matches the 2175 Å astronomical feature, but the bandwidths are broader (Fig. 1). This extra breadth may result from the ~10 times lower energy resolution of VEELS (relative to PAS) and from the grains' physical state. The subgrains within the IDPs are no longer free-floating in the ISM, and the extent of their solid-state modification during their ~4.5-billion-year post-ISM lifetimes is unknown. At the very least they may have undergone significant aggregation into larger (50- to 500-nm-diameter) grains, and computer-modeling best fits to the astronomical 2175 Å feature are obtained with much smaller (<15-nm-diameter) grain sizes (*1*–*4*). Production of the interstellar 2175 Å feature is generally thought to be due to electronic transitions associated with the surfaces of small grains, and modification of these surfaces by aggregation, for example, is expected to alter the spectral profile of the feature (*1*, *22*). Finally, all IDPs have been pulse heated to >350°C during atmospheric entry (*23*) where organic components and –OH-bearing grains (e.g., GEMS) are particularly susceptible to modification.

IR spectroscopy of selected areas rich in carbonaceous material in our IDPs indicates that carbonyl (C=O) groups are the likely carrier of much of the oxygen in the organic fractions (Fig. 4). The average bulk carbon content of IDPs is ~12 wt. % (*21*), at least half of which is hydrocarbons. About 10% of the carbon is bonded to oxygen, either in carbonyl (C=O) groups or as aromatic chromophores bound to hydroxyl (−OH) groups (*19*, *21*, *24*). Multiple VEELS measurements sampling these regions show that the 5.7-eV feature strength correlates with the O/C ratio (Fig. 3, A to E). This suggests that the 5.7-eV feature produced by the carbonaceous subgrains in IDPs may be due to organic molecules (e.g., PAHs) with

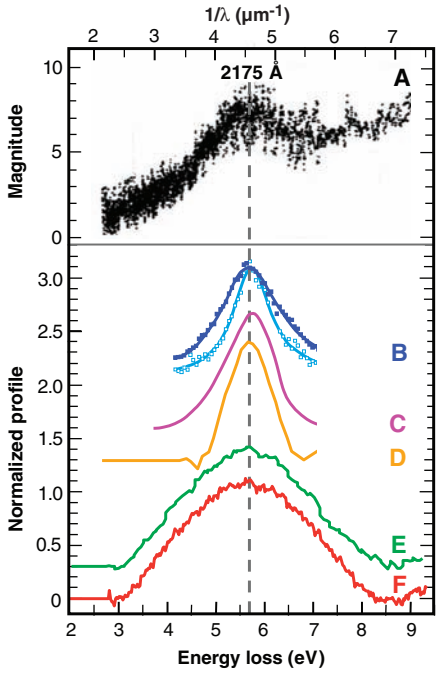

Fig. 1. Comparison of astronomical UV extinction features with laboratory UV and VEELS features. (**A**) The 2175 Å interstellar extinction feature from two stars ζ and ε Persei (*5*). (**B**) Broadest (ζ Oph) and narrowest (HD 93028) profiles from 45 stars (*6*). (**C**) Photoabsorption spectrum from partially recrystallized hydroxylated amorphous magnesium silicate (*8*). (**D**) VEELS spectrum from (electron) irradiation-damaged talc ($Mg_3Si_4O_{10}[OH]_2$). (**E**) VEELS spectrum from (organic) carbon in IDP L2047 D23. (**F**) VEELS spectrum from GEMS in W7013 E17.

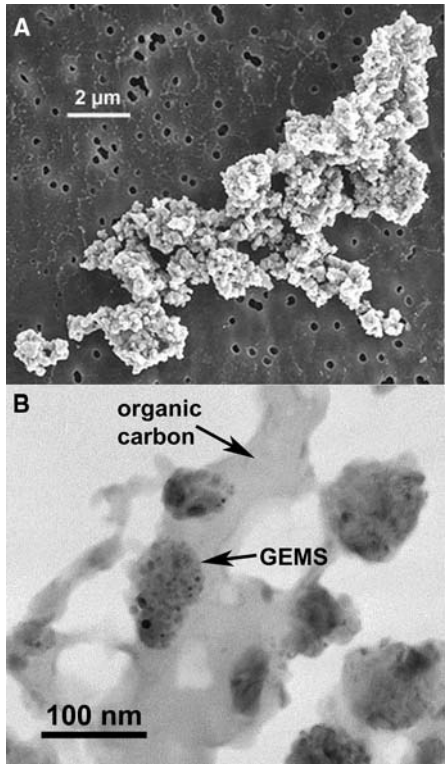

Fig. 2. (**A**) Secondary electron image of a typical chondritic IDP (RB12A). (**B**) A 200-keV brightfield transmission electron micrograph of organic carbon and GEMS within chondritic IDP L2009*E2.

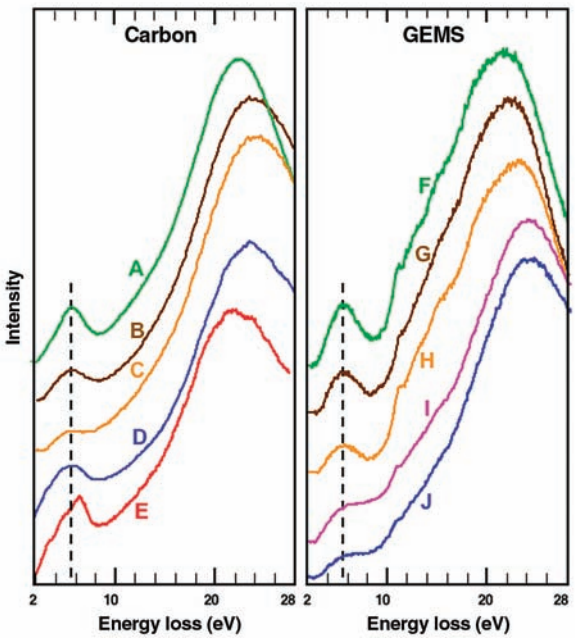

Fig. 3. VEELS spectra from subgrains in IDPs. (**Left**) Carbonaceous grains: (**A**) L2036-G16, ~500-nm-diameter grain, O/C = 0.41. (**B**) L2047 D23, ~800-nm-diameter grain with nonsolar $^{14}N/^{15}N$ ratio of 192 ± 4 (2σ), O/C = 0.07. (**C**) L2036-C18-F4, ~400-nm-diameter grain with correlated nonsolar $^{12}C/^{13}C$ = 80 ± 2.4 and $^{14}N/^{15}N$ = 135 ± 6.4 isotopic compositions, O/C = 0.09. (**D**) Pyrene. (**E**) 1-Pyrene carboxaldehyde. (**Right**) GEMS: (**F** to **H**) W7013E17 (three GEMS, each 400 to 500 nm in diameter): (F) O_{ex} = 29.0, (G) O_{ex} = 19.1, (H) O_{ex} = 12.3. (**I**) L2036-C24-I3, ~650-nm-diameter grain, nonsolar $^{16}O/^{17}O$ = 2262 ± 108, $^{16}O/^{18}O$ = 403 ± 8(9), O_{ex} = 2.3. (**J**) L2036-C18-F4, ~300-nm-diameter grain, O_{ex} = 1.6. Dashed lines indicate 5.7 eV. The weak 10.5-eV feature in GEMS spectra (right) is a silicate exciton [the position of which overlaps Lyman-α emission (*5*)].

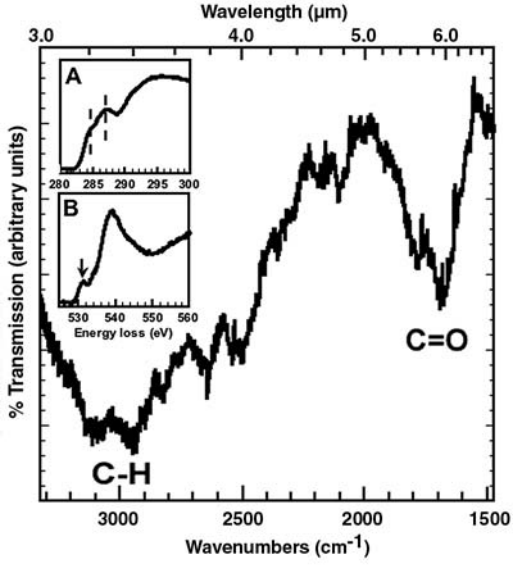

Fig. 4. IR spectrum from a ~9-μm² region of IDP L2036-G16 (Fig. 3A). Peaks at 2850 to 2960 cm^{-1} are due to aliphatic C-H stretch modes, and the peak at ~1720 cm^{-1} is due to carbonyl (C=O). Specimen thickness is <100 nm. (Insets) Electron energy-loss spectra recorded at 300 keV (no monochromator) of (**A**) carbon K-edge showing double π* edges (dashed lines) at ~285.0 eV and 286.5 eV consistent with carbonyl (C=O), and (**B**) oxygen K-edge showing a pre-edge at ~531 eV (arrow) associated with a 1s to π* transition of oxygen and also consistent with carbonyl (*19*, *21*, *24*).

carbonyl functionality. VEELS measurements of unsubstituted and carbonyl-substituted pyrene are consistent with this hypothesis (Figs. 3, D and E). Other measurements suggest that substituted PAHs are abundant in IDPs (*24*, *25*), and laboratory simulations of radiation processing in dense clouds have demonstrated that carbonyl groups can be added to PAHs under some interstellar conditions (*1*, *26*). Carbonyl-substituted PAHs are also present in carbonaceous chondrites (*27*).

Organic compounds may also be responsible for the 5.7-eV feature from GEMS because, in addition to being coated with carbon, some GEMS contain carbonaceous matter within their interiors (*28*). However, the strength of the GEMS 5.7-eV feature correlates with increasing O concentration (Fig. 3, F to J), implicating the inorganic O-rich glassy matrices. Stoichiometric excesses of O (O_{ex}) observed in GEMS may be due primarily to hydroxyl ions (OH^-) within their amorphous magnesium silicate matrices (*11*). Laboratory UV spectra of hydroxylated amorphous magnesium silicate particles exhibit an absorption feature at 2175 Å that matches both the central wavelength and bandwidth of the interstellar feature (*8*) and may be due to an electronic transition of hydroxyl ions in low-coordination sites (OH^-_{LC}) (Fig. 1C). In electron-irradiated talc ($Mg_3Si_4O_{10}[OH]_2$) (Fig. 1D), the feature is also likely due to OH^-_{LC} because talc degrades (amorphizes) rapidly under the electron beam. Similarly, most of the hydroxyl in GEMS is probably OH^-_{LC} because the glassy matrices are defect-rich from constant irradiation in space (*11*).

Carbonaceous and amorphous silicate grains exhibiting a 5.7-eV (2175 Å) UV feature in VEELS spectra have been identified in chondritic IDPs collected in the stratosphere. The species implicated as possible carriers for these features are carbonyl-containing molecules and hydroxylated amorphous silicates (GEMS). However, because carbonaceous material permeates some GEMS, molecular matter may be solely responsible for the 5.7-eV feature. Both materials may have been produced by irradiation processing of dust in the ISM. Before this study, amorphous silicates, but not carbonyl compounds, were suggested as potential carriers of the astronomical 2175 Å extinction feature. On the basis of our observations of IDPs, we cannot conclude that organic carbon and (hydroxylated) amorphous silicates are the only carriers of the astronomical feature. However, the identification of interstellar subgrains in IDPs (as evidenced by their isotopic compositions) that produce analogous features suggests that the carrier(s) of the interstellar feature may be present in IDPs. This finding provides new information for computational modeling, laboratory synthesis of analog grains, and laboratory (UV) photo-absorption measurements. It is also worth looking for a correlation of the interstellar 2175 Å feature with IR carbonyl and hydroxyl bands, although lines-of-sight suitable for detecting a strong 2175 Å feature are generally diffuse, whereas larger column densities are typically required for detection of weaker infrared bands. The presence of two potential carriers may bear on the variable bandwidth of the astronomical feature, with relative abundance or physical state of each component varying from one sight line to another. Amorphous silicates are ubiquitous throughout interstellar space, but oxidized (carbonyl-containing) PAHs have yet to be identified in the ISM, although they are indicated as a major product of irradiation of PAHs and are found in primitive meteorites. A variety of exotic carriers for the 2175 Å peak have been proposed, including nanodiamonds, carbon onions, and fullerenes (*1*–*4*). However, organic carbon and amorphous silicates are more abundant in interstellar space, and cosmically abundant carriers are needed to explain the ubiquity of the 2175 Å feature.

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9. Collected IDPs are typically <20 μm in diameter and are composed mostly of aggregated submicrometer grains including silicates, sulfides, metal, and inorganic and organic compounds (e.g., PAHs) (*10*). They are from comets and asteroids, they include the most chemically and isotopically primitive meteoritic materials available for laboratory investigations, and they are logical materials in which to look for carriers of the 2175 Å feature because they contain 10 to 100 times more interstellar material than the most primitive meteorites (*11*, *14*).
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Retinoic Acid Signaling Restricts the Cardiac Progenitor Pool

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Organogenesis begins with specification of a progenitor cell population, the size of which provides a foundation for the organ's final dimensions. Here, we present a new mechanism for regulating the number of progenitor cells by limiting their density within a competent region. We demonstrate that retinoic acid signaling restricts cardiac specification in the zebrafish embryo. Reduction of retinoic acid signaling causes formation of an excess of cardiomyocytes, via fate transformations that increase cardiac progenitor density within a multipotential zone. Thus, retinoic acid signaling creates a balance between cardiac and noncardiac identities, thereby refining the dimensions of the cardiac progenitor pool.

Generation of the proper number of organ progenitor cells is likely to involve interplay between inductive and repressive signaling pathways. Key inductive mechanisms have been identified for many organs, including the heart, but mechanisms for repressing progenitor fate assignment are poorly understood. Several factors, including Bmp2, Fgf8, Nodal, and Wnt11, are implicated in promoting the initial selection of myocardial progenitor cells from a multipotential population (*1*). Although convergence of inductive signals might be sufficient to delimit the number of progenitor cells, opposing signals could also be necessary to restrict myocardial specification. Prior studies have suggested mechanisms for inhibiting cardiomyocyte differentiation within the anterior lateral plate mesoderm (ALPM), by means of Notch signaling (*2*) or interactions with the notochord (*3*), but little is known about whether repressive pathways limit the initial assignment of myocardial identity.

We find that reduction of retinoic acid (RA) signaling causes formation of an excess of cardiomyocytes. The zebrafish mutation *neckless* (*nls*) disrupts function of the *retinaldehyde dehydrogenase 2* gene (*raldh2*), which controls a rate-limiting step in RA synthesis (*4*, *5*). *nls* mutants exhibit an increased number of cells expressing *nkx2.5*, a marker of the bilateral populations of precardiac mesoderm within the ALPM (Fig. 1A). Although *nkx2.5* expression appears expanded in anterior, posterior, and lateral directions (Fig. 1, A and B), we do not observe an increase in the overall size of the ALPM in *nls* mutants (fig. S1A). As myocardial differentiation proceeds, *nls* mutants exhibit a surplus of cardiomyocytes, identifiable by their expression of *cardiac myosin light chain 2* (*cmlc2*) (Fig. 1B; fig. S1B). Formation of this myocardial surplus depends on the conventional myocardial differentiation pathway (*1*), which requires the activity of the growth factor Fgf8 and the transcription factors Hand2 and Gata5 (fig. S2). Consistent with a repressive influence of RA on cardiomyocyte formation, exposure to the pan-retinoic acid receptor (RAR) antagonist BMS189453 (*6*, *7*) causes expansion of *nkx2.5* and *cmlc2* expression (Fig. 1, A and B). Conversely, exposure to exogenous RA results in a reduced number of cardiomyocytes (fig. S1C) (*8*). Together, these data demonstrate that cardiomyocyte population size within the ALPM is inversely related to the level of RA signaling.

The *raldh2* gene is expressed throughout early zebrafish embryogenesis (*4*, *5*): In the blastula, *raldh2* is found at the embryonic margin; during gastrulation, *raldh2* is in involuting mesendoderm; and, after gastrulation, *raldh2* is in both lateral and paraxial mesoderm. To investigate when RA influences cardiomyocyte number, we tested the effectiveness of BMS189453 during different time intervals, initiating exposure at various stages and later assessing *nkx2.5* or *cmlc2* expression (Fig. 1, C and D; fig. S3). Addition of BMS189453 before gastrulation [40% epiboly, 5 hours post fertilization (hpf)] causes a myocardial expansion, whereas addition of BMS189453 during gastrulation (75% epiboly, 8 hpf) results in a more modest increase.

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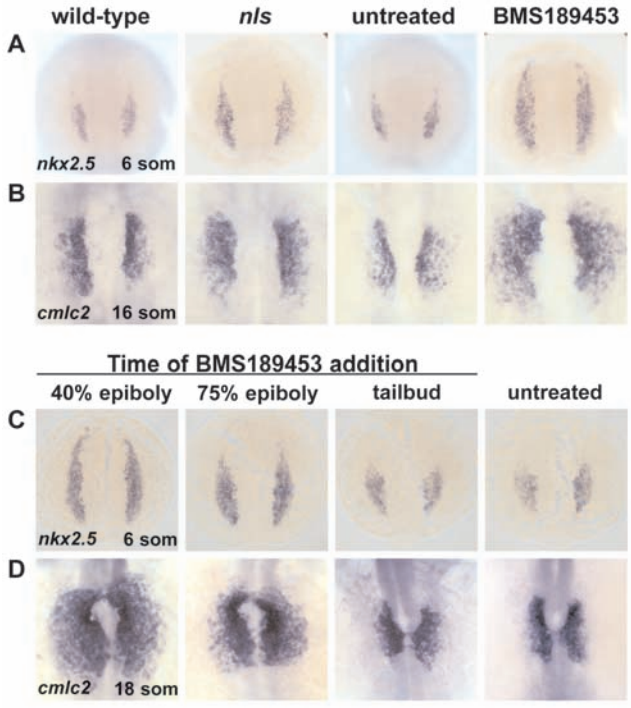

Fig. 1. Cardiomyocyte number increases when RA signaling is reduced. Expression of (**A** and **C**) *nkx2.5* and (**B** and **D**) *cmlc2*, at indicated somite (som) stages. Dorsal views, anterior to the top, comparing wild-type and *nls* mutant siblings, as well as untreated and BMS189453-treated wild-type siblings. (A and B) RAR antagonism has a more potent effect than *raldh2* loss of function, consistent with the presence of low levels of RA in *raldh2* mutants (*5, 10, 18, 19*). (C and D) RA signaling is required before tailbud stage to prevent expansion of *nkx2.5* and *cmlc2* expression.

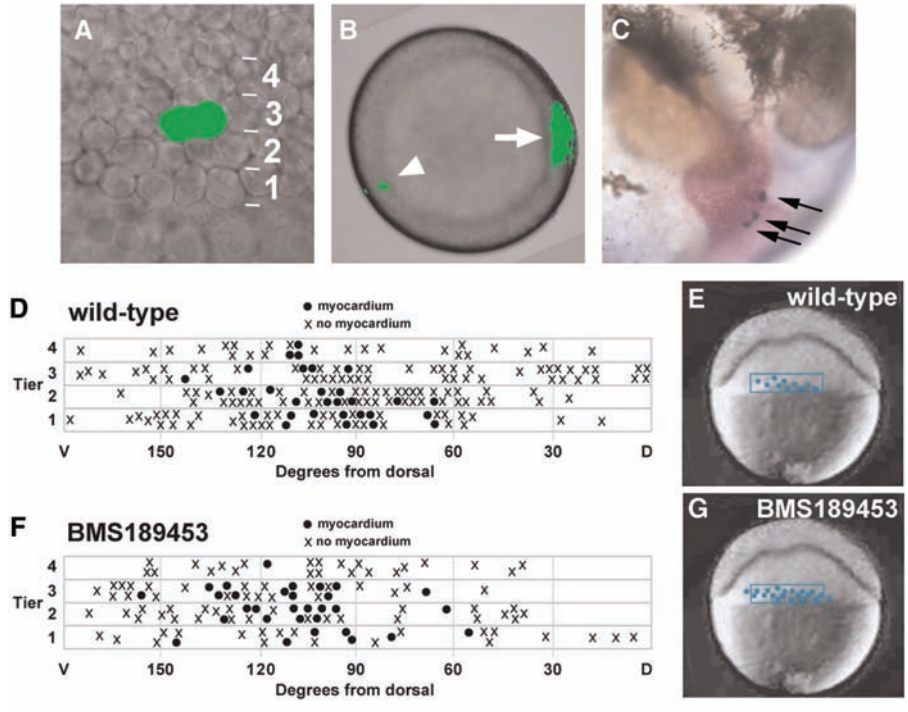

Fig. 2. Reduction of RA signaling increases myocardial progenitor density. (**A**) Lateral view, 40% epiboly, animal pole to the top. Latitude is measured in tiers (white bars and numerals), defined as cell diameters from the embryonic margin (*9*). Here, we labeled two neighboring blastomeres (green) in tier 3. (**B**) Animal view, dorsal to the right. Longitude is measured as angular distance along the embryonic circumference, with the dorsal midline [marked by the center of *Tg(gsc:gfp)* expression; arrow] defined as 0° (*9*). Here, labeled cells (arrowhead) are 153° to 160° from dorsal. (**C**) Frontal view at 44 hpf, head to the top. Here, we detected three labeled cardiomyocytes (blue cells, arrows; pink, *cmlc2* expression). (**D** and **F**) Myocardial fate maps in (D) wild-type and (F) BMS189453-treated embryos. Each symbol represents the location of labeled blastomeres in an individual experiment and indicates whether these cells contributed to the myocardium. (**E** and **G**) Schematics depicting myocardial progenitor distribution in the zebrafish blastula. Reduction of RA signaling increases the number of blastomeres in the LMZ (blue box) that become myocardial progenitors (blue dots). Images in (D) and (E) adapted from (*9*).

In contrast, adding BMS189453 at the end of gastrulation (tailbud, 10 hpf) does not alter total cardiomyocyte number. Therefore, RA signaling is required during gastrulation for its effects on cardiomyocyte population size.

Because of the early influence of RA on cardiomyocyte formation, we hypothesized that inhibition of RA signaling creates a myocardial surplus through fate transformation, rather than through myocardial proliferation. To test this model, we generated a myocardial fate map in BMS189453-treated embryos. By adapting a technique that we have used in wild-type embryos (*9*), we examined the frequency with which cardiomyocytes originate from particular locations within the blastula. Using laser-mediated activation of caged fluorescein (*6*), we labeled selected blastomeres at 40% epiboly. We recorded the positions of labeled blastomeres using latitude and longitude coordinates (Fig. 2, A and B) (*9*) and then treated embryos with BMS189453. Later, we evaluated the fates of labeled cells, with an emphasis on their cardiac contributions (Fig. 2C). In 60 of our 120 labeling experiments (Fig. 2F), labeled blastomeres were located in the lateral marginal zone (LMZ). This zone, defined as the first three tiers of blastomeres between 60° and 140° from the dorsal midline, is the most common region of origin for myocardial progenitors in wild-type embryos (Fig. 2D) (*9*).

Fate map comparisons (Fig. 2, D and F) reveal that RA affects the number of LMZ blastomeres that become myocardial progenitors. In both wild-type and BMS189453-treated embryos, myocardial progenitors are found throughout the LMZ (Fig. 2, D and F). However, in BMS189453-treated embryos, LMZ blastomeres generate myocardial progenitors twice as often as they do in wild-type embryos [labeled cardiomyocytes detected in 45% (27 out of 60) of BMS189453-treated embryos (Fig. 2F) versus 20% (20 out of 100) of comparable wild-type embryos (Fig. 2D) (*9*); binomial test, $P < 0.05$]. This substantial increase in progenitor density is accompanied by a slight expansion of the region containing myocardial progenitors; in BMS189453-treated embryos, we occasionally detect myocardial progenitors just outside of the dorsoventral boundaries of their typical wild-type locations (Fig. 2, D and F). Although BMS189453-treated embryos appear to contain an increased number of myocardial progenitors, the individual progenitors each seem to produce the same number of cardiomyocytes as their wild-type counterparts. On the assumption that only one progenitor is labeled in each experimental embryo (*9*), the average myocardial contribution per progenitor is 4.3 cardiomyocytes in wild-type embryos (*9*) and 4.8 cardiomyocytes

in BMS189453-treated embryos (table S1) (t test, $P > 0.2$). Therefore, reduction of RA signaling does not appear to affect cardiomyocyte proliferation during the stages examined. Altogether, the fate map data demonstrate that reduction of RA signaling causes a fate transformation that generates an excess of myocardial progenitors.

Formation of extra myocardial progenitors within the LMZ is likely to occur at the expense of another progenitor population. Some LMZ lineages seem unaffected by inhibition of RA signaling; for example, we labeled endocardial progenitors in 26% (26 out of 100) of wild-type embryos (*9*) and 25% (15 out of 60) of BMS189453-treated embryos (table S2). In contrast, other LMZ lineages (*9*) appear considerably diminished when RA signaling is reduced, including pancreas (*10*), pharyngeal pouches (*4*), and pectoral fin mesenchyme (*4*, *5*). All of these cell types are sensitive to RA signaling during the same time interval when RA influences myocardial progenitors (*5*, *10*) (fig. S3). The differential effects of reducing RA signaling during gastrulation suggest that RA influences the balance between cardiac and noncardiac progenitor populations that arise from within the LMZ (Fig. 2, E and G).

Taken together, our data indicate that RA signaling has a potent repressive role during cardiac specification. It is evident that this early role of RA is one of many functions for this signaling pathway during cardiogenesis; previously established roles for RA include influences on cardiac chamber identity (*8*, *11*–*13*), terminal myocardial differentiation (*14*), cardiac looping (*12*, *15*), and ventricular maturation and growth (*12*, *16*, *17*). Beyond uncovering an additional role for RA, our data also suggest a new means by which RA regulates fate assignment. Several previous studies, including analyses of the zebrafish hindbrain (*4*, *5*) and gut (*10*), have suggested that RA has a posteriorizing influence during the establishment of anterior-posterior coordinates. However, reduction of RA signaling does not appear to cause a unidirectional shift in the location of myocardial progenitors (Fig. 2). Instead of defining spatial boundaries for myocardial specification relative to the embryonic axes, RA signaling seems to impose limits on the density of myocardial progenitor cells intermingled within an eligible population. We propose that RA, synthesized within or near the LMZ (*4*, *5*), biases fate assignments in this area, restricting the opportunities for becoming myocardial. Thus, the repressive function of RA during cardiac specification provides an example of a previously unappreciated mode of selecting organ progenitors from within a multipotential pool.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/307/5707/247/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 and S2
References and Notes

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Global Circumnavigations: Tracking Year-Round Ranges of Nonbreeding Albatrosses

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Although albatrosses are paradigms of oceanic specialization, their foraging areas and migration routes when not breeding remain essentially unknown. Our continuous remote tracking of 22 adult gray-headed albatrosses for over 30 bird-years reveals three distinct strategies: (i) Stay in breeding home range; (ii) make return migrations to a specific area of the southwest Indian Ocean; and (iii) make one or more global circumnavigations (the fastest in just 46 days). The consistencies in patterns, routes, and timings offer the first hope of identifying areas of critical habitat for nonbreeding albatrosses, wherein appropriate management of longline fisheries might alleviate the plight of the world's most threatened family of birds.

Albatrosses are the world's most threatened family of birds (with 19 of 21 taxa on the International Union for the Conservation of Nature Red List). Recent catastrophic population decreases are mainly a consequence of incidental mortality in longline and trawl fisheries (*1*). Knowledge of the at-sea distribution of albatrosses is thus critical to their conservation, yet few data from birds of known status and provenance are available outside the breeding season. Regular successive recoveries have been made off eastern Australia of wandering albatrosses (*Diomedea exulans*) banded at South Georgia (54°00′S, 38°03′W), showing that some birds migrate great distances, but more recent tracking data from the same species breeding in the Indian Ocean indicate that this population is much more sedentary during the nonbreeding period (*2*–*4*). Satellite tracking of postbreeding birds of other species has confirmed several long-distance migrations (*5*, *6*), but the brevity of these studies and the small samples mean that range and behavior during the nonbreeding season is, by comparison, very little known.

We deployed leg-mounted light level loggers (*7*) on 47 gray-headed albatrosses (*Thalassarche chrystostoma*) that were rearing chicks at the end of the austral summer (April 1999) at Bird Island, South Georgia. This species breeds biennially when successful, and 35 of the loggers were retrieved between September and November 2000, of which 22 provided complete data throughout the approximately 18-month interval between breeding events (corresponding to 11,034 bird-days) and 13 failed to download.

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Subsequent data processing provided two locations per day with a probable mean accuracy ± SD of 186 ± 114 km (*7*), apart from a variable period around the equinoxes when latitude estimates are unreliable.

Three overall migration strategies were identified: (i) residence in the southwest Atlantic and adjacent areas in an extended version of the breeding season home range (*5*, *8*), also indicated by previous sightings at the colony of banded birds in their sabbatical year; (ii) return migrations to winter in habitats known to be used by other albatrosses in the Indian Ocean; and (iii) one or more global circumnavigations with foraging in areas and habitats used in options (i) and (ii) and also in additional staging areas in the Indian and Pacific Oceans (Fig. 1 and fig. S1). Investigated in more detail, these broad strategies apparently include different combinations of a limited number of consistent seasonal migration patterns [supporting online material (SOM) text]. There was a degree of sexual segregation: Females were much more likely to show a restricted range, whereas most males performed at least one circumpolar navigation.

We recorded 15 round-the-world journeys by 12 birds (3 of which made double journeys), all in an easterly direction. Any bird that moved beyond the Kerguelen archipelago (49°20′S, 70°20′E) completed such a trip, this area being the eastward limit for a simple return migration to and from South Georgia. There was high consistency in both the timing and choice of route, especially in winter. These circumnavigations included some extraordinary examples of albatross flight performance. Typical journeys from South Georgia to the southwest Indian Ocean took 6.2 days at 950 km per day; the leg to the southwest Pacific lasted 13.2 days at 950 km per day, and the last leg back to South Georgia 10.3 days at 750 km per day. Without stopping, a complete circumnavigation of the Southern Ocean could, in theory, be completed in 30 days; this provides a context for the exceptional performance of the bird that achieved this in just 46 days.

Our results show that (i) there were at least three distinct migration strategies; (ii) individuals consistently exploited the same general staging areas in succeeding winters; (iii) despite the apparent flexibility inherent in a nonbreeding season that lasts 18 months, the timing of migratory journeys was generally well synchronized and of consistent duration, and the routes were typically very similar both at the individual level and within strategies; and (iv) all individuals used the breeding season home range in the Atlantic for at least part of the nonbreeding summer.

Very little is known about environmental, hormonal, or physiological factors influencing the selection of different migration patterns, routes, and staging areas. However, the apparent sexual segregation could result from niche divergence mediated by differences in flight performance (*8*). In addition, the observed spatial and temporal consistencies suggest that correct timing of events is particularly important, although the cues that trigger movements are unknown. Although all staging areas coincide with habitats either known or likely (given bathymetric and hydrographic features) to be of importance to albatrosses (fig. S1), there are indications that South Georgia birds may use these areas when local albatrosses (especially conspecifics and congeners) are not breeding (and therefore potentially absent). Similarly, tracking studies during the breeding season suggest little or no spatial overlap between adjacent colonies or populations (*9*, *10*).

The relatively small size of the key staging areas and restricted amplitude of the migration routes suggests that it may be possible to identify critical habitats. This might help mitigate interactions with threatening processes (particularly fisheries) and also to identify biodiversity hot spots in pelagic marine systems. Indeed, compared with congeners, the gray-headed albatross has a greater propensity for aggregation at frontal systems and other mesoscale oceanographic features, rather than in shelf waters (*4*), which makes it a potentially valuable indicator species for biodiversity hot spots in pelagic habitats. In comparing its year-round distribution (fig. S1) with data on tuna longline fishing effort (*11*), only in the southwest Indian Ocean does a core albatross staging area coincide with one of the five or six principal fishing areas exploited over the last decade. However, their migration routes traverse most of the key tuna fishing areas south of 30°S, with the exception of the Tasman Sea. This reinforces the message that protecting albatross and petrel species requires appropriate mitigation measures to be used in longline fisheries throughout all oceans south of 30°S.

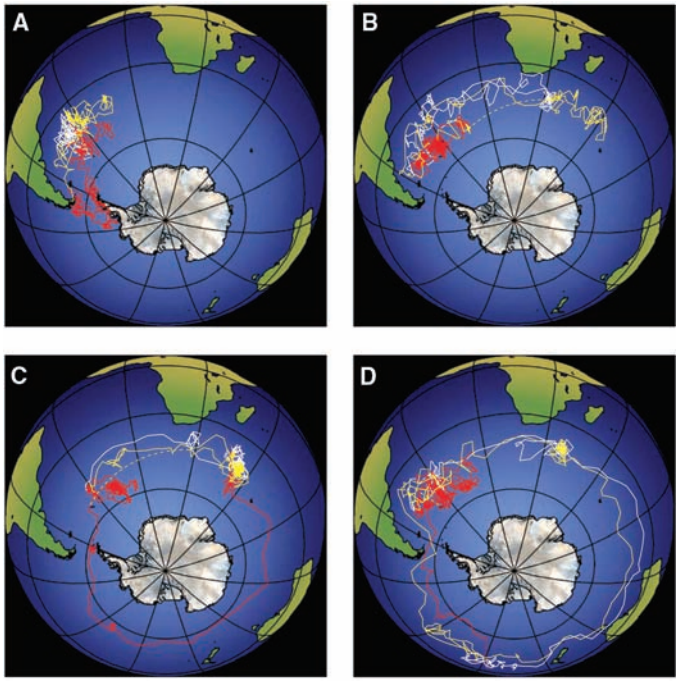

Fig. 1. (**A** to **D**) Representative migration routes of four gray-headed albatrosses in the 18 months between breeding attempts (SOM text). White, first winter; red, summer; yellow, second winter. Dotted lines link locations obtained before and after the equinox.

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Fig. S1
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No Transcription-Translation Feedback in Circadian Rhythm of KaiC Phosphorylation

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An autoregulatory transcription-translation feedback loop is thought to be essential in generating circadian rhythms in any model organism. In the cyanobacterium *Synechococcus elongatus*, the essential clock protein KaiC is proposed to form this type of transcriptional negative feedback. Nevertheless, we demonstrate here temperature-compensated, robust circadian cycling of KaiC phosphorylation even without *kaiBC* messenger RNA accumulation under continuous dark conditions. This rhythm persisted in the presence of a transcription or translation inhibitor. Moreover, kinetic profiles in the ratio of KaiC autophosphorylation-dephosphorylation were also temperature compensated in vitro. Thus, the cyanobacterial clock can keep time independent of de novo transcription and translation processes.

Circadian clocks are ubiquitous endogenous biological timing processes that adapt to daily alterations in environmental conditions in bacteria, fungi, plants, and animals. In most model organisms, the core process that generates and maintains self-sustaining oscillations is thought to be a ~24-hour-period transcriptional and translational oscillatory process (TTO) based on negative feedback regulation of clock genes (*1*, *2*). Cyanobacteria are the simplest organisms known to exhibit circadian rhythms. In the cyanobacterium *Synechococcus elongatus* PCC 7942, three adjacent genes at the *kai* locus (*kaiA*, *kaiB*, and *kaiC*) are essential for circadian function (*3*). The amounts of accumulated *kaiBC* operon mRNA and KaiC protein oscillate in a circadian manner with a ~6-hour delay under continuous light (LL), reminiscent of the temporal profiles of negative elements in eukaryotic clock systems (*4*). Continuous overexpression of *kaiC* nullifies circadian rhythms and abolishes *kaiBC* expression, whereas a transient increase in KaiC sets the phase of the rhythm (*3*, *4*). These observations led to the cyanobacterial TTO model for explaining prokaryotic circadian rhythm generation, in which KaiC and KaiA negatively and positively regulate *kaiBC* transcription, respectively (*3*). In the cyanobacterial circadian system, essentially all the promoter activities on the genome show such rhythms (*5*). Moreover, the functional target of the Kai clock system may not be clock-gene–specific but could affect genome-wide gene expression, such as chromosomal DNA conformation (*6*–*8*). In this revised model, rhythmic *kaiBC* transcription is still thought to be essential for producing and maintaining a self-sustaining basic oscillation underlying circadian genome-wide global expression control, consistent with the widely accepted TTO model.

Because *Synechococcus* is an obligate photoautotroph, most of its metabolic activities, including total RNA and protein syntheses, are quickly suppressed when the cells are transferred to constant dark (DD) conditions (*9*). Nevertheless, dark treatments for 3 to 42 hours affected the phase of the rhythm less when the bacteria were returned to continuous light (LL) (*4*), which reveals that the clock can free-run during DD and thus contradicts the TTO model. Indeed, it has not yet been examined whether rhythmic *kaiBC* expression per se is truly essential for generating circadian rhythms. Thus, we examined *kaiA* and *kaiBC* mRNA accumulation profiles under DD conditions. Expression of *kaiBC* mRNA showed robust circadian rhythms during LL after a 12-hour dark treatment (Fig. 1, A and B). Conversely, when cells were transferred to DD from the light, the *kaiBC* mRNA was reduced within 4 hours to almost undetectable levels (less than 0.1% of the peak levels during LL)

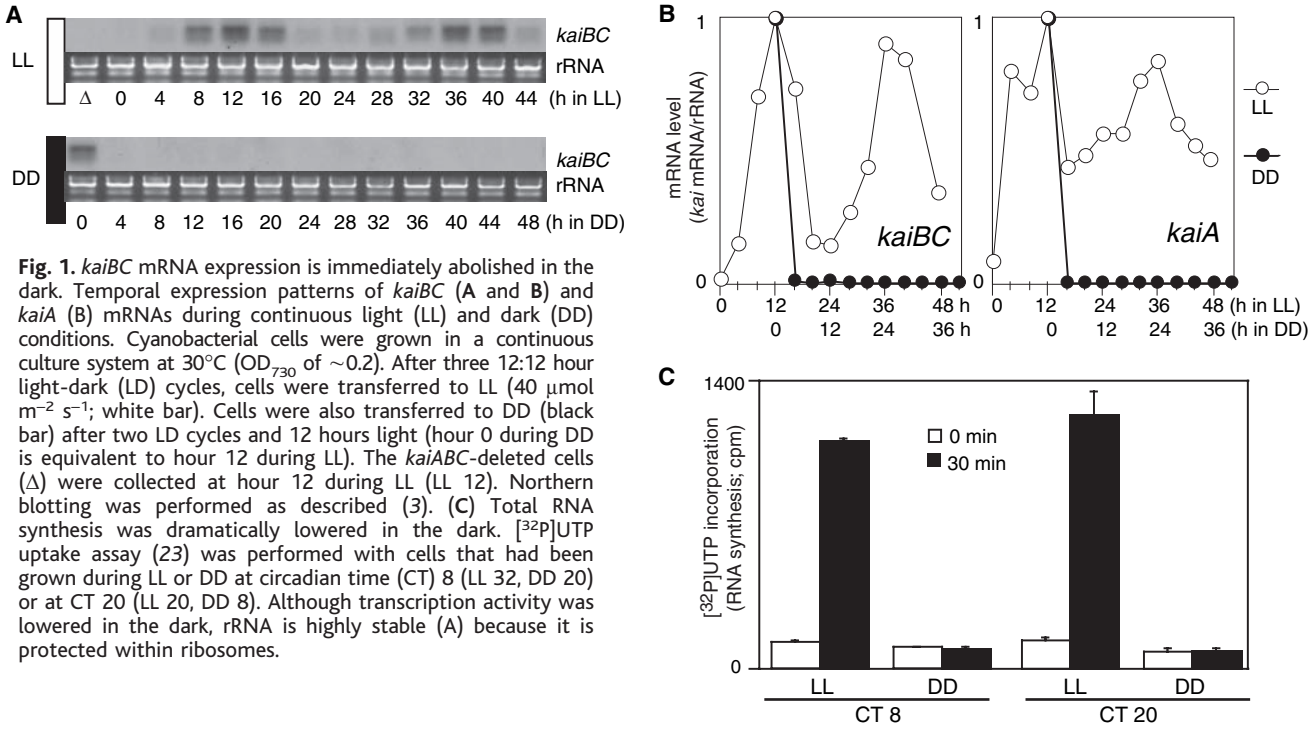

Fig. 1. *kaiBC* mRNA expression is immediately abolished in the dark. Temporal expression patterns of *kaiBC* (**A** and **B**) and *kaiA* (B) mRNAs during continuous light (LL) and dark (DD) conditions. Cyanobacterial cells were grown in a continuous culture system at 30°C (OD_{730} of ~0.2). After three 12:12 hour light-dark (LD) cycles, cells were transferred to LL (40 μmol m^{-2} s^{-1}; white bar). Cells were also transferred to DD (black bar) after two LD cycles and 12 hours light (hour 0 during DD is equivalent to hour 12 during LL). The *kaiABC*-deleted cells (Δ) were collected at hour 12 during LL (LL 12). Northern blotting was performed as described (*3*). (**C**) Total RNA synthesis was dramatically lowered in the dark. [^{32}P]UTP uptake assay (*23*) was performed with cells that had been grown during LL or DD at circadian time (CT) 8 (LL 32, DD 20) or at CT 20 (LL 20, DD 8). Although transcription activity was lowered in the dark, rRNA is highly stable (A) because it is protected within ribosomes.

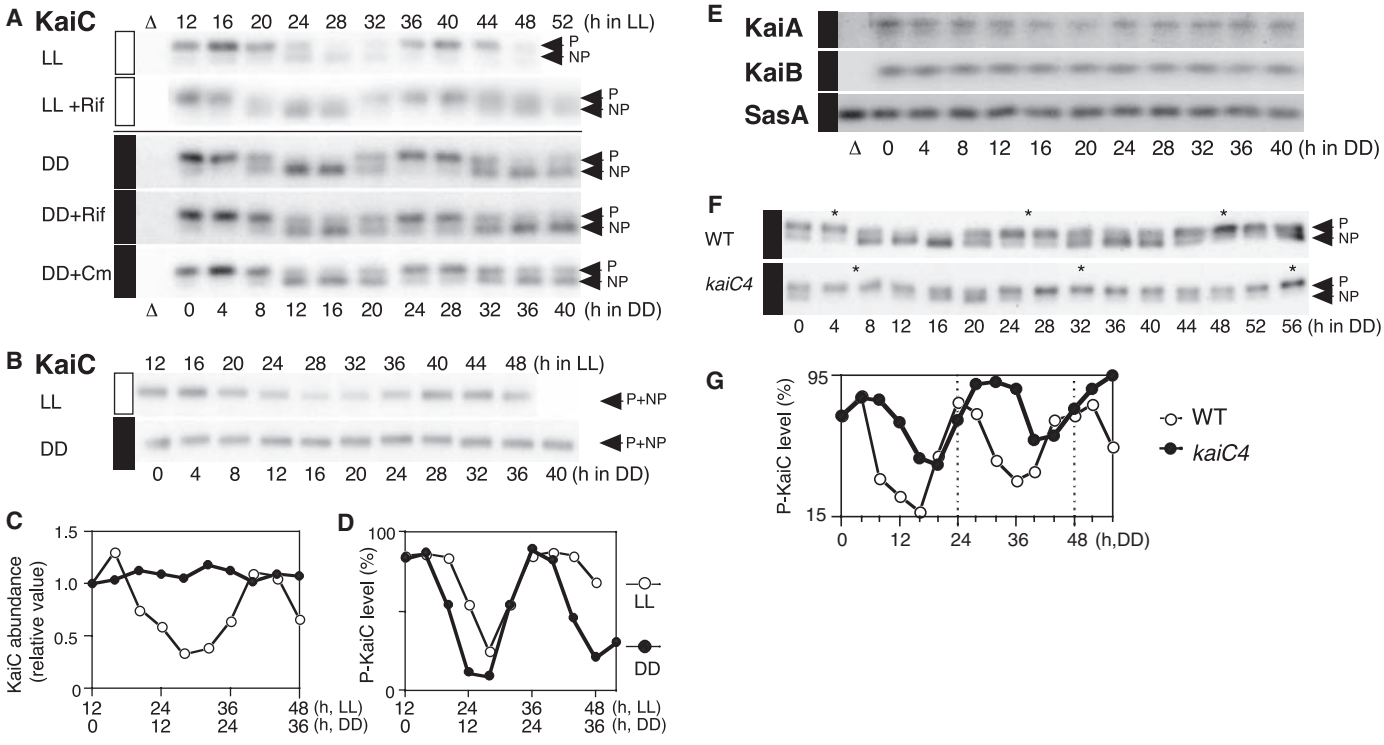

Fig. 2. KaiC phosphorylation rhythm without *kaiBC* mRNA accumulation. (**A**) Temporal profiles of KaiC accumulation and phosphorylation during LL and DD were examined by Western blot. Total protein (1 μg) prepared from cells was analyzed by immunoblotting with antiserum to KaiC on 10% SDS polyacrylamide gels (bisacrylamide:acrylamide = 1:149), as described previously (*10*). The upper and lower bands correspond to phosphorylated (P) and nonphosphorylated (NP) forms of KaiC, respectively. When Rifampicin (Rif) (200 μg/ml) and chloramphenicol (Cm) (400 μg/ml) were added to the cultures at hour 0 during DD (DD 0) or at hour 12 during LL (LL 12), the KaiC phosphorylation rhythm profiles were less affected. Representative data are shown. (**B**) The same protein samples of KaiC in LL or DD used in (A) were analyzed on 10% polyacrylamide gels (bisacrylamide:acrylamide = 2:73) to appear as single-band signals. (**C**) Quantification of KaiC by densitometry of the blots shown in (B). Values at times LL 12 or DD 0 were normalized to 1.0. (**D**) Percentages of the phosphorylated KaiC (P-KaiC) levels compared with the total KaiC levels in (A) are shown. (**E**) KaiA, KaiB, and SasA accumulation was also maintained at constant levels in the dark (Western blot). (**F**) The KaiC phosphorylation rhythm under DD in wild-type (WT) and *kaiC4* mutant strains (Western blot). Asterisks indicate the peaks of KaiC phosphorylation. (**G**) Densitometric analysis of (F).

(Fig. 1, A and B). All *kaiA* mRNA also disappeared (Fig. 1B). We confirmed that general transcription activity, as measured by the incorporation of [^{32}P]-UTP (uridine triphosphate) into RNA, was lowered to background levels, whereas active transcription was observed in the light (Fig. 1C). Therefore, de novo clock-gene expression and Kai protein syntheses, essential processes in the TTO model, must be shut off almost completely in DD.

Under LL conditions, KaiC shows not only a circadian rhythm of accumulation but also circadian changes in phosphorylation at Ser and Thr residues (*10*). Phosphorylation of KaiC is essential for circadian timing, because mutation of the phosphorylation sites abolishes circadian oscillations in *Synechococcus* (*11*, *12*).

To address how Kai proteins behave in the dark, we examined the accumulation profiles of the Kai proteins and the phosphorylation profile of KaiC under DD conditions. In contrast to the rhythmic KaiC accumulation in LL, KaiA, KaiB, and KaiC proteins were maintained at constant levels, with abolition of their rhythmic accumulation after transfer to DD (Fig. 2, A to C and E). Therefore, Kai proteins were more stable during DD than during LL (*13*). A KaiC-associating histidine kinase, SasA (*14*), also accumulated to a constant amount, as observed in LL (Fig. 2E). Even in the absence of the rhythmic accumulation of the Kai proteins and of detectable *kaiA* and *kaiBC* mRNAs, KaiC phosphorylation showed a robust circadian rhythm lasting at least 56 hours in DD, equivalent to that observed in LL (Fig. 2, A, F, and G). The KaiC phosphorylation rhythm in DD was less affected in the presence of excess amounts of a transcription or translation inhibitor (200 μg/ml rifampicin or 400 μg/ml chloramphenicol) (Fig. 2A). These inhibitors repressed >95% of total protein and RNA syntheses during LL at these concentrations (*15*, *16*). Therefore, in contrast to the widely accepted TTO model, the self-sustainable rhythm during DD can persist at the posttranslational level without any accompanying de novo synthesis of central clock-gene mRNAs or their encoded proteins. The *kaiC4* mutant strain that harbors a P236S substitution in KaiC lengthens the period of the *kaiBC* expression rhythm by 4 hours during LL (*3*). The phosphorylation rhythm in the dark was lengthened accordingly (Fig. 2, F and G). Therefore, the circadian period length appears precisely determined, even in the absence of transcriptional feedback. In addition, because the KaiC phosphorylation cycle in the presence of rifampicin was also observed during LL (Fig. 2A), *kaiBC* transcription is not necessary for generation of the posttranslational rhythm, regardless of LL or DD conditions.

The basic features of circadian rhythms include temperature and nutrient compensation such that the periods are similar at different ambient temperatures or with different nutritional supplementation (*1*). Because the KaiC phosphorylation rhythm is maintained with a ~24-hour period, even when metabolic activities are lowered during DD, the rhythm must be nutrient compensated. The period of KaiC phosphorylation cycling under DD was almost

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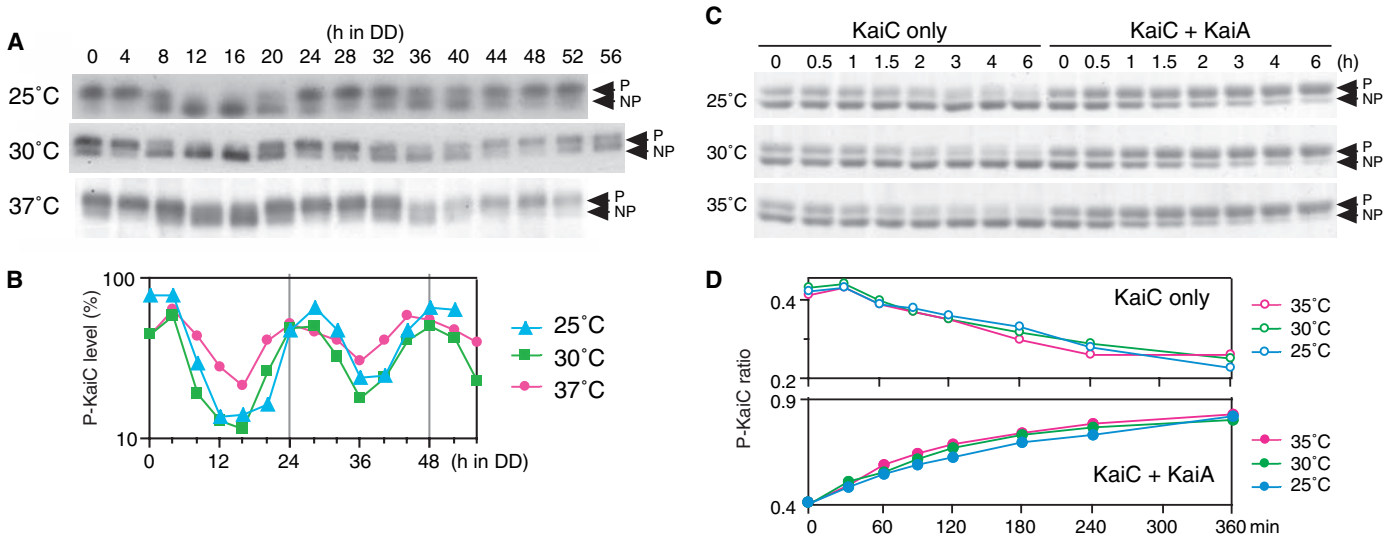

Fig. 3. Analysis of KaiC phosphorylation at different temperatures. (**A** and **B**) Western blot analysis. KaiC phosphorylation rhythms were examined under DD at 25°C, 30°C, and 37°C. Densitometric analysis of (A) is shown in (B). (**C** and **D**) In vitro KaiC-autokinase/autophosphatase activity at different temperatures. Recombinant KaiA and KaiC proteins were produced in *E. coli* and purified as described (*10*). In the presence (0.05 μg/μl) or absence of KaiA, KaiC (0.2 μg/μl) was incubated in a reaction buffer (50 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM ATP; pH 8.0) at 25°C, 30°C, and 35°C. Samples of the reaction mixtures were collected at the indicated times and subjected to SDS–polyacrylamide gel electrophoresis on 7.5% gels followed by Coomassie brilliant blue staining (C). Densitometric analysis of (C) is shown in (D), in which the ratio of P-KaiC to total KaiC is plotted.

the same at different temperatures (25°C, 30°C, and 37°C) (Fig. 3, A and B). Thus, the circadian oscillatory process during DD is temperature compensated.

KaiC phosphorylation in vivo is regulated by its own autokinase (*10*, *17*) and autophosphatase (*6*, *18*, *19*) activities. KaiA activates KaiC autophosphorylation (or inhibits KaiC autodephosphorylation), whereas KaiB negates KaiA's effect on KaiC both in vitro and in vivo (*6*, *10*, *18*, *19*). To characterize the autophosphorylation-dephosphorylation reactions of KaiC in vitro, recombinant KaiC protein was purified from *Escherichia coli* and incubated with adenosine triphosphate at different temperatures in the presence or absence of KaiA (25°C, 30°C, and 35°C). Prepared recombinant protein (time zero) appeared as phosphorylated and nonphosphorylated bands on SDS polyacrylamide gels (Fig. 3C). At 30°C, KaiC was almost completely dephosphorylated after 6 hours of incubation with ATP, whereas the addition of KaiA enhanced the phosphorylation of KaiC (Fig. 3C). The equilibrated ratio of phosphorylated and nonphosphorylated forms of KaiC, and the transition profiles to the equilibrium states, were less affected by different temperatures (25°C and 35°C) in the presence or absence of KaiA (Fig. 3, C and D) (*20*). These phenomena are probably caused by two antagonistic reactions—autophosphorylation and autodephosphorylation—each with a similar temperature coefficient regardless of the presence of KaiA. The temperature compensation of the net KaiC autophosphorylation and autodephosphorylation reactions in vitro is essentially due to the biochemical properties of the KaiC protein and does not require any other specific proteins or de novo synthesis of KaiC. Therefore, KaiC's temperature-insensitive autophosphorylation-dephosphorylation reaction rates may partly explain the temperature compensation of the KaiC phosphorylation cycle during DD and are critical processes for circadian timing.

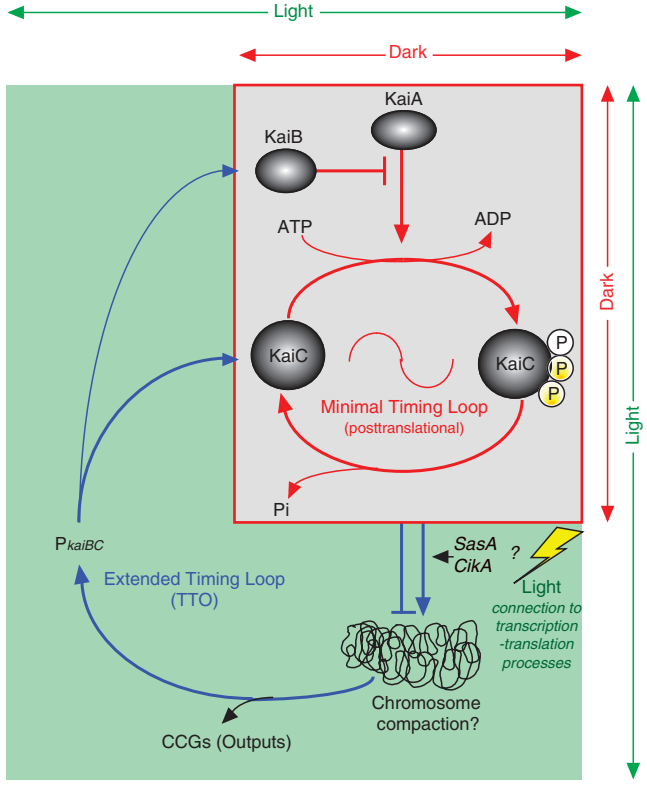

Fig. 4. A model for the posttranslational oscillator coupled with TTO. The KaiC phosphorylation cycle can be maintained in the dark as a minimal timing loop without transcription or translation (gray area). During LL, gene expression activated by an energy supply from photosynthesis expands the oscillation to the TTO form (green area). Two histidine kinases (SasA and CikA) (*24*) might be required to connect KaiC function to a process in the general transcription mechanism, such as chromosome superhelicity (*8*), which feeds back to the *kaiBC* promoter (P_{kaiBC}) activity and regulates output gene expression globally in a circadian manner. In the dark or under nutrition-limited conditions, the posttranslational oscillator may work as a "time memory" process to ensure robust circadian organization in *Synechococcus*. Pi and CCGs indicate phosphate and clock-controlled genes, respectively.

The KaiC phosphorylation rhythm in vivo is accompanied by formation of a series of KaiC complexes with KaiA and/or KaiB in a circadian fashion during LL (*18*, *21*). KaiA

stimulates phosphorylation of KaiC hexamer progressively, and then the phosphorylated KaiC hexamer forms a complex with KaiA. This KaiA binding likely triggers the binding of phosphorylated KaiC-KaiA complexes to KaiB, an attenuator of KaiC autophosphorylation, to shift KaiC from a phosphorylation-dominating state to a dephosphorylation-dominating state. Reduction in the KaiC phosphorylation level would, in turn, accelerate phosphorylation reactions and/or inhibit dephosphorylation with as-yet-unknown nonlinear processes, whereby both states alternate periodically. Such autoregulatory posttranslational dynamics of the Kai proteins would be the core to generating the temperature-compensated, self-sustaining KaiC phosphorylation cycle as a minimal circadian circuit during DD (Fig. 4). The genome-wide transcriptional control exerted by KaiC that occurs during LL (*6*, *7*) may connect to the minimal oscillator to form an extended feedback loop that further amplifies and stabilizes the posttranslational oscillatory process and serves as a major source of physiologically functional rhythm outputs (Fig. 4). Posttranslational biochemical oscillation would also be physiologically important as a "time memory" process to keep circadian rhythms ticking even in dark or metabolically limited (nonpermissive) conditions that severely perturb transcriptional or translational processes.

Robust oscillations of the phosphorylation of core clock proteins have been shown to be critical in these circadian systems in *Neurospora*, *Arabidopsis*, *Drosophila*, and mammals (*1*). These phosphorylations have been shown to affect stability, intracellular localization, and/or DNA-binding property of key clock components, and these cyclic changes are important to maintain robust cycling of the TTO processes (*1*). Moreover, constitutive induction of both *period* and *timeless* genes in *Drosophila* has been shown to be sufficient to produce cycling in the coding protein abundances (*22*), further supporting the critical role of posttranslational regulation in the eukaryotic clock system. We show here that the de novo synthesis of core clock elements is not an absolute requirement in allowing biochemical reactions to oscillate with a temperature-compensated circadian period.

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13. Results shown here contradict the previous interpretation of Xu *et al.* (*4*), who suggested the persistence of cyclical KaiC induction even in the dark. However, according to recent retrospective comments by the above authors (*6*), nonphosphorylated KaiC in their Western blots (*4*) was obscured by high nonspecific background, and KaiC phosphorylation was not known at that time. Therefore, together with our data, we suggest that even their original data (*4*) may not have demonstrated cyclic KaiC protein synthesis but may have reflected the KaiC phosphorylation rhythm during DD. In addition, the calculated half-life of KaiC in the presence of 400 μg/ml chloramphenicol is ~10 hours in LL (*15*), whereas that in DD is >80 hours (Fig. 2, A to C).
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CX_3CR1-Mediated Dendritic Cell Access to the Intestinal Lumen and Bacterial Clearance

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Dendritic cells (DCs) and macrophages are critical to innate and adaptive immunity to the intestinal bacterial microbiota. Here, we identify a myeloid-derived mucosal DC in mice, which populates the entire lamina propria of the small intestine. Lamina propria DCs were found to depend on the chemokine receptor CX_3CR1 to form transepithelial dendrites, which enable the cells to directly sample luminal antigens. CX_3CR1 was also found to control the clearance of entero-invasive pathogens by DCs. Thus, CX_3CR1-dependent processes, which control host interactions of specialized DCs with commensal and pathogenic bacteria, may regulate immunological tolerance and inflammation.

Antigen-presenting dendritic cells (DCs) play a crucial role in establishing immunity to pathogens, tolerance to self-antigens, and the induction of organ-specific autoimmunity (*1*–*5*). Through their interactions with antigen-specific T lymphocytes, DCs induce adaptive immune responses and are also critical in controlling tissue inflammation. At mucosal interfaces, DCs constantly survey and process commensal bacteria and pathogens as a first step in the development of immunity, and recent studies have suggested that distinct DC subsets achieve this through diverse means (*6*–*8*). In addition to acquiring antigens in the lamina propria, intestinal DCs located below specialized intestinal epithelial cells (IECs), called M cells, also detect incoming pathogens. Furthermore, intestinal DCs transport bacteria and capture proteins from the gut lumen (*9*). Recent evidence has suggested that intestinal DCs also directly monitor the content of the intestinal lumen by either entering or extending dendrites into the epithelium (*10*, *11*). However, both the molecular basis and the physiological relevance of this process remain unclear.

To characterize the intestinal mononuclear phagocytes that are responsible for the recognition of intestinal pathogens, we used mice in which one or both copies of the gene encoding the chemokine receptor CX_3CR1 (*cx₃cr1*) was replaced with green fluorescent protein (GFP) reporter cDNA (*12*, *13*). In these mice, GFP expression is under the control of the CX_3CR1 promoter and, consequently, heterozygous mice ($cx_3cr1^{GFP/+}$) express both the receptor and GFP, whereas homozygous mice ($cx_3cr1^{GFP/GFP}$) express GFP but are CX_3CR1-deficient. As a result, GFP expression can be used to identify, isolate, and characterize intestinal cell populations that express the chemokine receptor. These cell populations are targets for CX_3CL1/fractalkine, a transmembrane chemokine expressed at the surface of IECs and endothelial cells in the intestine (*14*, *15*). In earlier studies, we found GFP and CX_3CR1 expressed in circulating monocytes and their progeny within tissues (*16*). Although CX_3CR1 was found to contribute to the migration of blood monocytes into tissues, no phenotypic or functional differences were detected in the peripheral lymphoid system between heterozygous and wild-type mice (*16*).

Examination of gut-associated tissues from $cx_3cr1^{GFP/+}$ and $cx_3cr1^{GFP/GFP}$ mice by fluorescence microscopy revealed GFP-positive mononuclear cells in the lamina propria, Peyer's patches (PPs), and mesenteric lymph nodes (MLNs) (Fig. 1A and fig. S1). Upon isolation, lamina propria leukocytes from $cx_3cr1^{GFP/+}$ and $cx_3cr1^{GFP/GFP}$ mice were found to represent different developmental stages of immature and mature DCs, which expressed the proteins CD11c and CD11b (Fig. 1B and fig. S1). Lamina propria DCs from $cx_3cr1^{GFP/+}$ and $cx_3cr1^{GFP/GFP}$ mice also expressed major histocompatibility complex (MHC) class II, CD80, and CD86 proteins at levels that were comparable to those found in DCs isolated from wild-type mice (fig. S1). Lamina propria CX_3CR1-positive DCs lacked CCR6 expression, in contrast to DC subsets that had been associated with PPs (*17*–*20*). Expression of the CX_3CR1 ligand CX_3CL1 was at its highest level in IECs that were isolated

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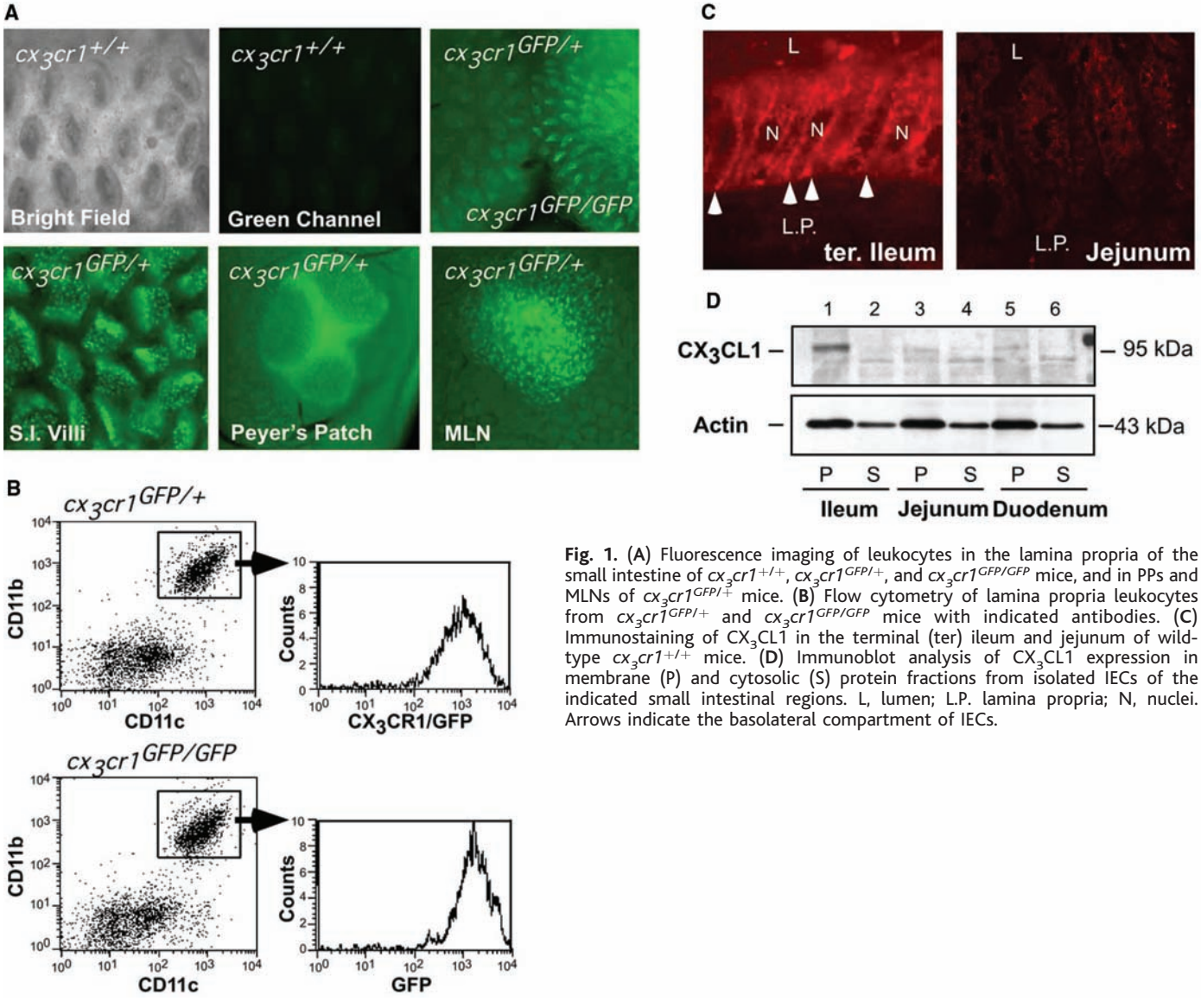

Fig. 1. (**A**) Fluorescence imaging of leukocytes in the lamina propria of the small intestine of $cx_3cr1^{+/+}$, $cx_3cr1^{GFP/+}$, and $cx_3cr1^{GFP/GFP}$ mice, and in PPs and MLNs of $cx_3cr1^{GFP/+}$ mice. (**B**) Flow cytometry of lamina propria leukocytes from $cx_3cr1^{GFP/+}$ and $cx_3cr1^{GFP/GFP}$ mice with indicated antibodies. (**C**) Immunostaining of CX_3CL1 in the terminal (ter) ileum and jejunum of wild-type $cx_3cr1^{+/+}$ mice. (**D**) Immunoblot analysis of CX_3CL1 expression in membrane (P) and cytosolic (S) protein fractions from isolated IECs of the indicated small intestinal regions. L, lumen; L.P. lamina propria; N, nuclei. Arrows indicate the basolateral compartment of IECs.

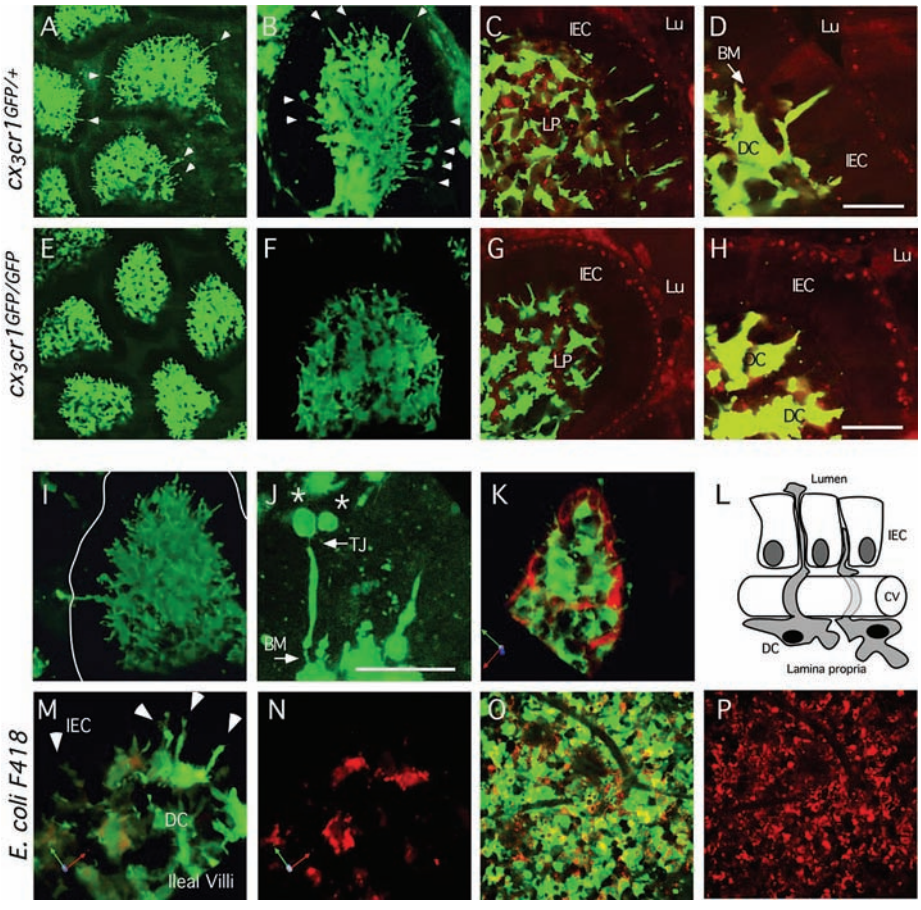

Fig. 2. (**A** to **D**) Confocal microscopic analysis of ileal mucosa of $cx_3cr1^{GFP/+}$ and (**E** to **H**) $cx_3cr1^{GFP/GFP}$ mice. (B) 3D tissue reconstruction of small intestinal villi from $cx_3cr1^{GFP/+}$ and (F) $cx_3cr1^{GFP/GFP}$ mice. In (C), (D), (G), and (H) the epithelium was counterstained with orange CMTMR [5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine]. (**I**) 3D analysis of a villus or (**J**) of the apical part of a villus in $cx_3cr1^{GFP/+}$ mice. (**K**) 3D analysis of a villus in $cx_3cr1^{GFP/+}$ mice after staining of the microvascular system with Texas Red Dextran. (**L**) Model representation of the morphologic relationship of DCs, microvasculature, and IECs. (**M** and **N**) 3D analysis of a villus in $cx_3cr1^{GFP/+}$ mice after oral administration of DsRed2-expressing *E. coli*. The GFP signal from CX_3CR1-positive DCs was removed in (N) to reveal the presence of *E. coli*. (**O**) Merged dual- or (**P**) single-channel confocal microscopy of a PP in $cx_3cr1^{GFP/+}$ mice after uptake of DsRed2-expressing *E. coli*. BM, basal membrane; Lu, lumen; LP, lamina propria; TJ, tight junctions; CV, capillary vessels. Asterisks indicate luminal dendritic cell compartments. Arrow heads indicate transepithilial dendrites in (A), (B), and (M). Scale bars, 10 μm.

Table 1. CX_3CR1-dependent uptake of nonpathogenic *E. coli* into intestine-associated lymphoid tissues. The number of colony-forming units (CFUs) of DsRed2-expressing *E. coli* F-18 in MLNs and PPs of $cx_3cr1^{+/+}$, $cx_3cr1^{GFP/+}$, and $cx_3cr1^{GFP/GFP}$ mice (n = 5 mice in each group) is shown 18 hours after oral administration.

Mice	Mesenteric lymph node (CFU per g of tissue)	Peyer's patch (CFU per g of tissue)
$cx_3cr1^{+/+}$	912 ± 256	97,444 ± 53,690
$cx_3cr1^{GFP/+}$	1,664 ± 1,300	102,221 ± 68,380
$cx_3cr1^{GFP/GFP}$	None	79,377 ± 76,076

from the terminal ileum, where the ligand was detected in basolateral membrane compartments (Fig. 1, C and D).

Living intestinal and lymphoid tissues were examined using confocal microscopy to determine the distribution and morphology of intestinal DCs and their interaction with IECs. In $cx_3cr1^{GFP/+}$ mice, lamina propria DCs extended dendrites into and through the intestinal epithelium (Fig. 2, A to D). However, these transepithelial dendrites were only observed in villi of the terminal ileum and were absent in the duodenum, jejunum, proximal ileum, and mid-ileum of the same mice. In contrast, ileal villi in $cx_3cr1^{GFP/GFP}$ mice lacked these intraepithelial DC extensions, suggesting that their formation is dependent on a CX_3CR1-mediated interaction with IECs (Fig. 2, E to H). In $cx_3cr1^{GFP/+}$ mice, 1.74 ± 0.2 intraepithelial dendrites were observed per villus, but only 0.07 ± 0.02 were observed per villus in the $cx_3cr1^{GFP/GFP}$mice (n = 40 villi from each of three mice). Nevertheless, DCs in the lamina propria of $cx_3cr1^{GFP/GFP}$ mice retained the ability to form dendrites, although these were confined to the lamina propria, which is consistent with their impaired capacity to traverse the epithelial cell monolayer (Fig. 2, G and H).

The complex structure of transepithelial processes was revealed by three-dimensional (3D) tissue reconstructions of intestinal villi. Intraepithelial dendrites were seen to originate in the lamina propria, either from the DCs that directly underlie the epithelium (Fig. 2, D and I) or from the DCs that are separated from the epithelium by the capillary blood vessel system (Fig. 2K). Intraepithelial dendrites extended through the IEC monolayer to end in mono- or multiglobular structures outside the epithelium (Fig. 2, I and J). The diameter of dendrites was reduced to 0.2 to 0.5 μm in regions where they crossed the basal membrane and the epithelial tight junction, possibly as a means of limiting disturbances to intestinal barrier function. This analysis suggests that the interaction of CX_3CL1 expressed on IECs with CX_3CR1 on intestinal DCs has a critical role in transepithelial dendrite formation.

Because the extension of transepithelial dendrites has been shown to be involved in luminal antigen sampling (*11*), we examined the uptake of commensal and enteropathogenic bacteria by lamina propria DCs in $cx_3cr1^{GFP/+}$ and $cx_3cr1^{GFP/GFP}$ mice. In $cx_3cr1^{GFP/+}$ mice, DsRed2-labeled nonpathogenic *Escherichia coli* were observed in DCs that extended dendrites into the IEC layer and in DCs within interfolicular regions (IFRs). DsRed2-expressing *E. coli* could be cultured from isolated PPs and MLNs of these mice (Fig. 2 and Table 1). In contrast, the uptake of *E. coli* into the lamina propria was impaired in the $cx_3cr1^{GFP/GFP}$ mice, and *E. coli* could not be cultured from isolated MLNs of these mice (Table 1). However, equal numbers of *E. coli* were recovered from PPs of $cx_3cr1^{GFP/GFP}$, $cx_3cr1^{GFP/+}$, and $cx_3cr1^{+/+}$ mice, indicating that bacterial sampling by M cells remained intact in these animals. The sampling defect in $cx_3cr1^{GFP/GFP}$ mice may thus specifically affect lamina propria DCs that are responsible for the transport of commensal bacteria to the MLNs.

To determine whether bacterial sampling and transport have consequences for the ability of $cx_3cr1^{GFP/GFP}$ mice to cope with bacterial infection, we examined mice infected with the enteroinvasive pathogen *Salmonella typhimurium*. Dendrite formation in $cx_3cr1^{GFP/+}$ mice was enhanced (3.8 ± 1.1 dendrites per villus, n = 20 villi from four

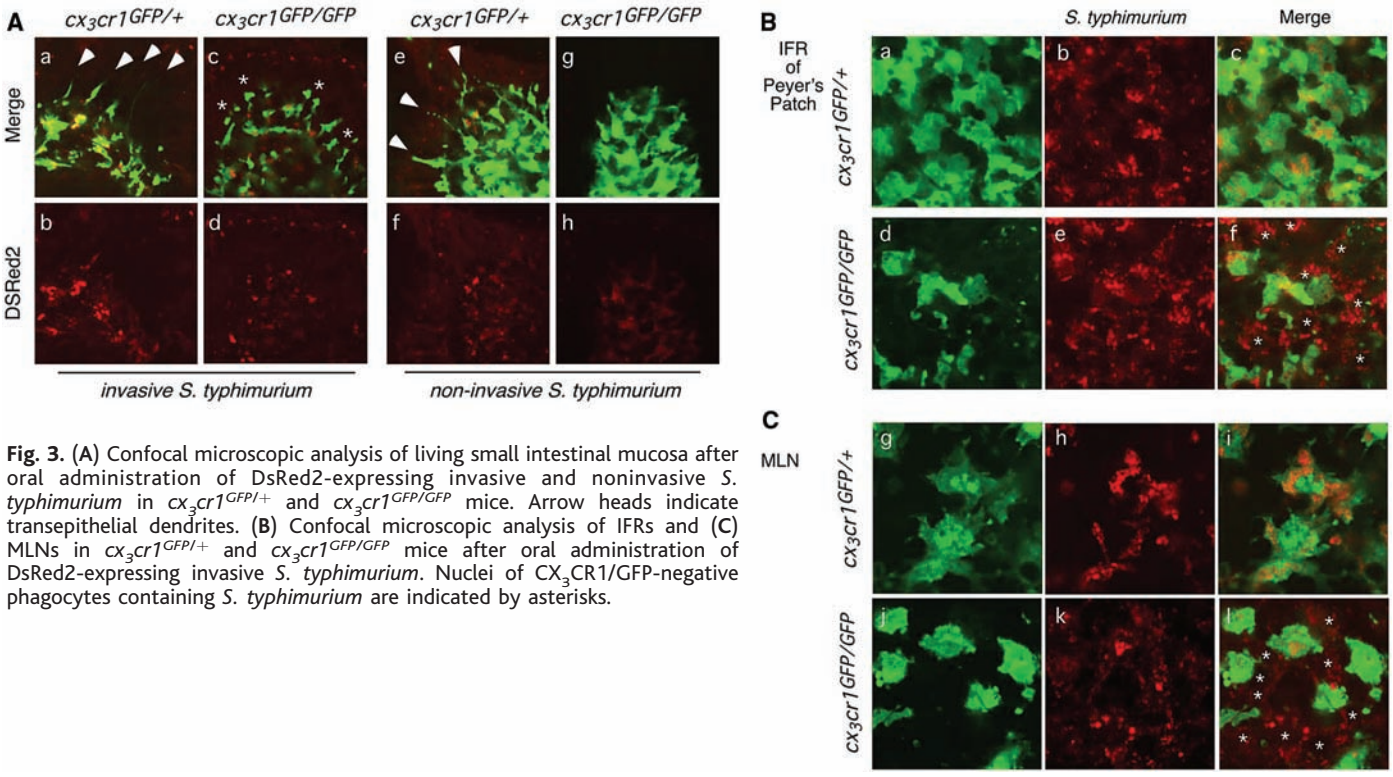

Fig. 3. (**A**) Confocal microscopic analysis of living small intestinal mucosa after oral administration of DsRed2-expressing invasive and noninvasive *S. typhimurium* in $cx_3cr1^{GFP/+}$ and $cx_3cr1^{GFP/GFP}$ mice. Arrow heads indicate transepithelial dendrites. (**B**) Confocal microscopic analysis of IFRs and (**C**) MLNs in $cx_3cr1^{GFP/+}$ and $cx_3cr1^{GFP/GFP}$ mice after oral administration of DsRed2-expressing invasive *S. typhimurium*. Nuclei of CX_3CR1/GFP-negative phagocytes containing *S. typhimurium* are indicated by asterisks.

mice in response to *S. typhimurium* infection, whereas the number of transepithelial processes did not increase in infected $cx_3cr1^{GFP/GFP}$ mice (0.08 ± 0.01 dendrites per villus, $n = 20$ villi from four mice) (Fig. 3A, panels a to d). In response to *S. typhimurium* infection, DCs in $cx_3cr1^{GFP/GFP}$ mice formed only globular structures at the basal surface of IECs, which failed to cross the epithelium (Fig. 3A, panel c). An invasion-deficient mutant of *S. typhimurium* was observed in DCs that extended transepithelial dendrites in the terminal small intestine 12 hours after oral administration in $cx_3cr1^{GFP/+}$ but not in $cx_3cr1^{GFP/GFP}$ mice (Fig. 3A, panels e to h). In contrast, invasive *S. typhimurium* traversed the intestinal epithelium in the $cx_3cr1^{GFP/GFP}$ mice independently of dendrite formation, and were subsequently phagocytosed by lamina propria DCs (Fig. 3A, panels c and d). Uptake of *S. typhimurium* by lamina propria DCs was also observed in MHC class II–GFP transgenic mice (fig. S2). In $cx_3cr1^{GFP/+}$ mice, invasive *S. typhimurium* were found confined to CX_3CR1/GFP–positive DCs in the IFRs and MLNs. In contrast, large numbers of bacteria were observed in a GFP-negative phagocyte subset in the $cx_3cr1^{GFP/GFP}$ mice (Fig. 3, B and C). This suggests that in the absence of CX_3CR1 expression, an additional lamina propria phagocyte subset may have been recruited to defend against invasive *S. typhimurium* but was unable to compensate for the loss of DC function in the $cx_3cr1^{GFP/GFP}$ mice. The number of invasive and noninvasive *S. typhimurium* that were recovered from CX_3CR1-deficient lamina propria DCs after in vitro uptake was reduced when compared to DCs from cx_3cr1^{GFP+} and wild-type control mice (Fig. 4, A and B). These results suggest that CX_3CR1 is required for the control

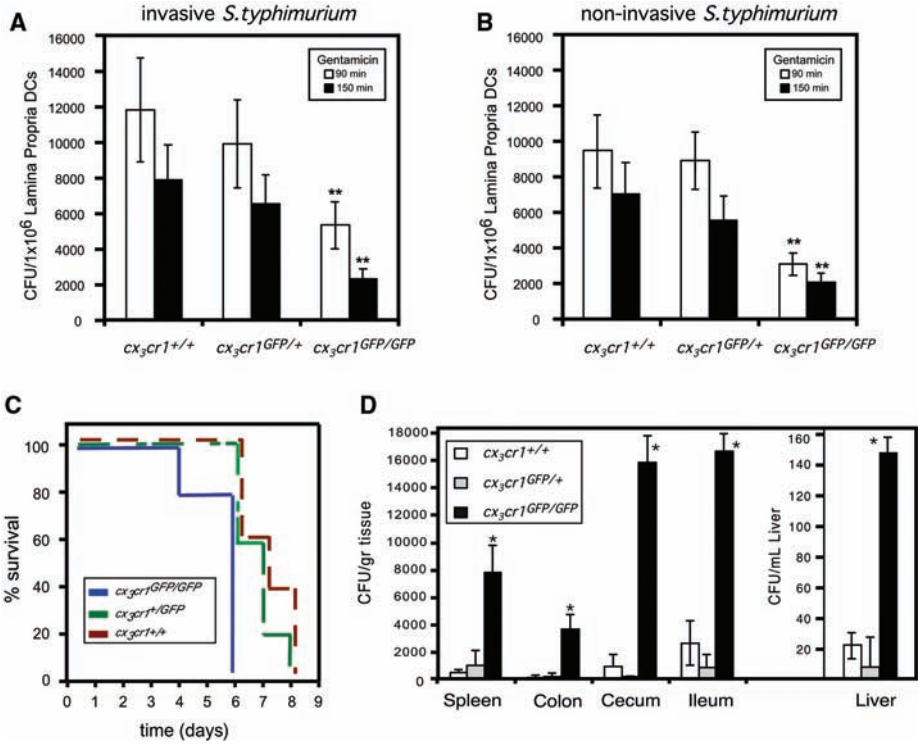

Fig. 4. (**A** and **B**) In vitro uptake of invasive and noninvasive *S. typhimurium* by lamina propria DCs after isolation from $cx_3cr1^{GFP/+}$, $cx_3cr1^{GFP/GFP}$, and $cx_3cr1^{+/+}$mice (**$P < 0.001$, $cx_3cr1^{GFP/+}$ DCs compared to $cx_3cr1^{GFP/GFP}$ DCs). (**C**) Survival of indicated mouse strains after oral infection with 10^9 *S. typhimurium*. (**D**) Organ load with *S. typhimurium* in $cx_3cr1^{GFP/+}$ and $cx_3cr1^{GFP/GFP}$ mice and their $cx_3cr1^{+/+}$ littermates (*$P < 0.001$, $cx_3cr1^{GFP/+}$ mice compared to $cx_3cr1^{GFP/GFP}$mice).

of the uptake of *S. typhimurium* by DCs within the intestinal mucosa. Most likely as a consequence of impaired bacterial sampling by DCs, $cx_3cr1^{GFP/GFP}$ mice displayed enhanced susceptibility to *S. typhimurium* infection. Thus, they succumbed within 6 days of oral administration of 1×10^9 bacteria and displayed significantly higher bacterial loads in their organs, compared with the relative resistance of their $cx_3cr1^{GFP/+}$ and wild-type counterparts (Fig. 4, C and D). Delayed pathogen uptake by DCs from the lumen and the lamina propria may thus form the mechanistic foundation for the impaired antibacterial defense in CX_3CR1-deficient mice.

We have demonstrated an extensive intestinal DC network that serves as a gateway for the uptake and transport of the intestinal microbiota. We have identified and characterized CX_3CR1-positive lamina propria DCs, a major component of this system, which are capable of taking up bacteria by way of transepithelial dendrites in order to provide defense against pathogenic microorganisms. CX_3CR1 deficiency results in a defect of lamina propria DCs that impairs the sampling of bacteria from the intestinal lumen and impedes their ability to take up invasive pathogens in vitro. The intrinsic functional programs and subspecifications of CX_3CR1-positive and -negative DCs in mucosal innate and adaptive immune responses will need to be further defined. Nevertheless, CX_3CR1-dependent regulation of DCs appears to provide a central mechanism for the control of the mucosal defense against entero-invasive bacteria.

The interaction of DCs with the intestinal microbiota by the way of CX_3CR1-dependent transepithelial dendrites could activate an innate immune pathway that protects the mucosa from pathogenic bacteria. The formation of these dendrites may be linked to the immature phenotype of lamina propria DCs and associated with their phagocytic function. Their use thus constitutes a mechanism by which DCs could take up intestinal antigens, which is distinct from previously established systems involving M cells. We propose that the CX_3CR1-dependent and the M cell–dependent systems could thus be associated with specific DC subsets. It will be important to determine whether such networks operate synergistically as redundant systems or if they have distinct functions in the recognition of commensal and pathogenic bacteria. Luminal sampling by CX_3CR1-positive DCs occurs by globular structures formed at the end of transepithelial dendrites, which could serve as luminal sensors for the mucosal immune system to continually monitor intestinal content. Characterization of the surface components that facilitate antigen uptake through this specialized cellular compartment may aid in developing strategies to prevent bacterial and viral pathogens from co-opting this route during infection. Furthermore, targeting of antigens to transepithelial dendrites could be used to directly engage the function of intestinal CX_3CR1-positive DCs in vaccine development.

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www.sciencemag.org/cgi/content/full/307/5707/254/DC1
Materials and Methods
Figs. S1 and S2
References
Movies S1 to S4

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Anticonvulsant Medications Extend Worm Life-Span

Kimberley Evason, Cheng Huang, Idella Yamben, Douglas F. Covey, Kerry Kornfeld*

Genetic studies have elucidated mechanisms that regulate aging, but there has been little progress in identifying drugs that delay aging. Here, we report that ethosuximide, trimethadione, and 3,3-diethyl-2-pyrrolidinone increase mean and maximum life-span of *Caenorhabditis elegans* and delay age-related declines of physiological processes, indicating that these compounds retard the aging process. These compounds, two of which are approved for human use, are anticonvulsants that modulate neural activity. These compounds also regulated neuromuscular activity in nematodes. These findings suggest that the life-span–extending activity of these compounds is related to the anticonvulsant activity and implicate neural activity in the regulation of aging.

Aging is characterized by widespread degenerative changes. Although treatments for aging would be desirable, the development of such treatments is challenging. Approaches based on rational design require information about the aging process, but little information is currently available. Approaches based on random screens of potential treatments require relevant and feasible assays of aging, but the time and effort necessary to measure aging are substantial obstacles.

To address these challenges, we exploited the *C. elegans* model system. These animals age rapidly, and many processes are conserved between nematodes and vertebrates, including aspects of the aging process (*1*). To identify compounds that delay aging, we assayed 19 drugs from a variety of functional or structural classes that have known effects on human physiology (*2*). We reasoned that such compounds might have an undiscovered effect on aging. For each drug, hermaphrodites were cultured with three different concentrations from before fertilization until death, and the adult life-span [fourth larval (L4) stage to death] of about 50 animals was measured. To focus on aging, we excluded dead worms that displayed internally hatched progeny, an extruded gonad, or desiccation due to crawling off the agar.

Ethosuximide had the greatest effect on adult life-span, extending mean adult life-span from 16.7 to 19.6 days (17% increase) (Fig. 1B and Table 1). A dose-response analysis revealed that worms cultured with external concentrations of 2 and 4 mg/ml ethosuxi-

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mide displayed the largest extensions of mean life-span; lower concentrations caused smaller extensions, whereas higher concentrations caused toxicity and reduced life-span (*3*). This effect was temperature sensitive; ethosuximide extended mean life-span by 35% at 15°C, 17% at 20°C, and insignificantly at 25°C (*3*).

Ethosuximide is a small heterocyclic ring compound that prevents absence seizures in humans and has been a preferred drug for treating this disorder since its introduction in the 1950s (*4*, *5*) (Fig. 1A). An important question is whether the anticonvulsant activity in humans and the life-span extension activity in worms have a similar mechanism. If this is the case, then other drugs with similar structures and anticonvulsant activity might also affect life-span. Trimethadione and 3,3-diethyl-2-pyrrolidinone (DEABL) have anticonvulsant activity and structures similar to that of ethosuximide (*4*, *6*) (Fig. 1A). Trimethadione is approved for human use and the treatment of absence seizures. DEABL is not used to treat humans. Both compounds caused significant extensions of mean and maximum life-span (Fig. 1, C and D, and Table 1). Trimethadione caused the largest extension of mean (47%) and maximum (57%) life-span of the three compounds. Succinimide, similar in structure but lacking in anticonvulsant activity in vertebrates, did not extend life-span (Fig. 1A and Table 1). These findings suggest that ethosuximide, trimethadione, and DEABL may extend life-span by a similar mechanism that may be related to the mechanism of anticonvulsant activity.

For the treatment of seizures, the therapeutic range of ethosuximide in humans is 40 to 100 μg/ml (*5*). Worms cultured with an external concentration of 2 mg/ml ethosuximide had an internal concentration (±SD) of 30.5 ± 22.2 μg/ml. This value is near the therapeutic range, suggesting that the anticonvulsants may have similar targets in worms and humans.

To determine the developmental stage at which the drugs function to extend life-span, trimethadione was administered from fertilization until the L4 stage or from the L4 stage until death. Exposure to trimethadione only during embryonic and larval development had no effect on life-span. In contrast, exposure to trimethadione only during adulthood caused a significant extension of mean life-span (24%) (Fig. 1D and Table 1).

To determine whether these drugs delay age-related declines of physiological processes, we analyzed self-fertile reproduction, body movement, and pharyngeal pumping. The declines of pharyngeal pumping and body movement are positively correlated with each

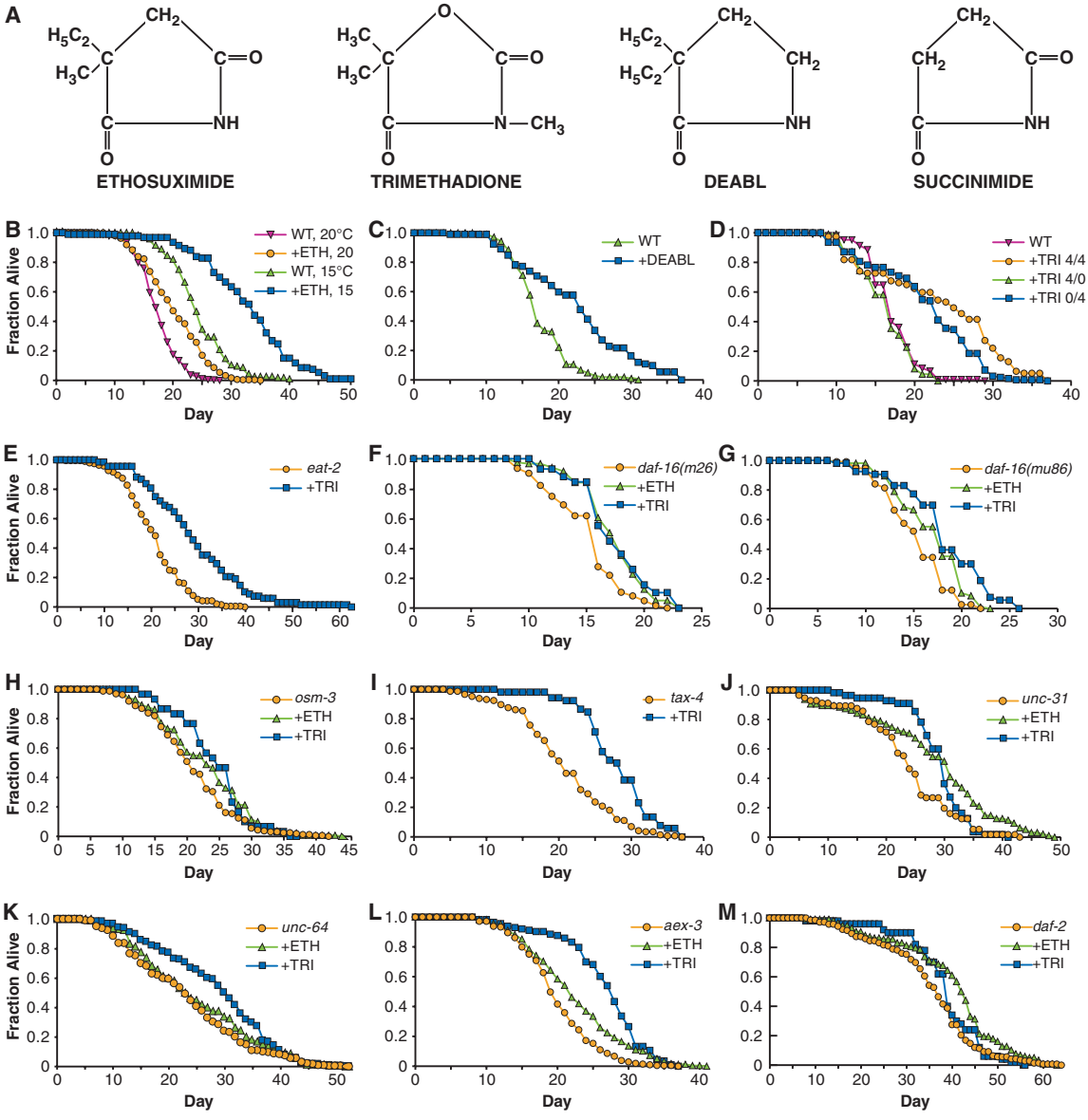


Fig. 1. Anticonvulsants extend adult worm life-span. (**A**) Compounds. (**B** to **M**) Hermaphrodite survival of [(B) to (D)] wild type (WT), (E) *eat-2(ad465)*, (F) *daf-16(m26)* (G) *daf-16(mu86)*, (H) *osm-3(p802)*, (I) *tax-4(p678)*, (J) *unc-31(e928)*, (K) *unc-64(e246)*, (L) *aex-3(ad418)*, and (M) *daf-2(e1370)*. Worms were exposed to ethosuximde (+ETH), DEABL (+DEABL), trimethadione from fertilization until L4 (+TRI 4/0), trimethadione from L4 until death (+TRI 0/4), or trimethadione from fertilization until death (+TRI 4/4 and +TRI). Dosages are shown in Table 1.

other and with life-span (*7*). The decline of self-fertile reproduction is not correlated with life-span, suggesting that this age-related change is regulated independently (*7*). Treatments with ethosuximide and/or trimethadione significantly extended the span of time that animals displayed fast body movement, fast pharyngeal pumping, and any pharyngeal pumping (Fig. 2, B to D and F). Neither compound significantly extended the span of time that animals displayed self-fertile reproduction (Fig. 2, A and F). These measurements can be used to define stages of aging (*7*). Both compounds extended Stage II, the postreproductive period characterized by vigorous activity (Fig. 2E). Trimethadione also extended Stage IV, the terminal phase characterized by minimal activity. These findings indicate that ethosuximide and trimethadione delay the aging process.

Several genetic and environmental manipulations can extend *C. elegans* life-span. To investigate the relationships between the anticonvulsants and these regulators of aging, we examined the effect of combining two treatments. Worms cultured on nonpathogenic *Bacillus subtilis* or ultraviolet (UV)–irradiated *E. coli* display an extended life-span (*8*, *9*). Trimethadione extended the life-span of worms cultured on *B. subtilis* and UV-irradiated *E. coli* (Table 1), indicating that the primary mechanism of the anticonvulsant life-span extension is not a reduction of bacterial pathogenicity.

Nutrient limitation extends life-span and can be caused by a mutation of the *eat-2* gene that is important for pharyngeal pumping (*1*, *10*, *11*). Trimethadione significantly extended the life-span of *eat-2* mutants (42%) (Fig. 1E and Table 1), indicating that the primary mechanism of life-span extension is not nutrient limitation. Furthermore, wild-type animals treated with ethosuximide or trimethadione were not nutrient limited, because they displayed normal pharyngeal pumping, food ingestion, and body morphology (they did not appear thin or starved), and they produced an approximately normal number of progeny (*3*).

An insulin-like signaling pathway regulates *C. elegans* life-span. This pathway requires the function of sensory neurons that may mediate the release of an insulin-like ligand, the *daf-2* insulin-like growth factor (IGF) receptor gene, and a signal transduction cascade that regulates the *daf-16* forkhead transcription factor gene. Loss-of-function *daf-16* mutations reduce life-span and suppress the life-span extensions caused by mutations in upstream signaling pathway genes such as *daf-2* (*12*). Treatment with ethosuximide or trimethadione significantly extended the life-span of two loss-of-function mutants, *daf-16(m26)* (16%) and *daf-16(mu86)* (11 to 21%) (Fig. 1, F and G, and Table 1), although the percentage change caused by trimethadione was less than that in wild-type animals (47%). These results indicate that part of the anticonvulsant action is independent of *daf-16*. Part of the anticonvulsant action may require *daf-16*. However, the reduced effect of trimethadione is consistent with other possibilities, such as deleterious consequences of combining a mutation and a drug that both cause pleiotropic effects (*13*).

Life-span extension is caused by loss-of-function mutations of genes important for the function of sensory neurons (*osm-3* and *tax-4*), for neurotransmission (*unc-31*, *unc-64*, and *aex-3*), and for transmission of the insulin-like signal (*daf-2*) (*12*, *14*, *15*) (Table 1). Ethosuximide and/or trimethadione significantly increased the life-span of *osm-3*, *tax-4*, *unc-31*, *unc-64*, *aex-3*, and *daf-2* loss-of-function mutants from 8 to 36% (Fig. 1, H to M, and Table 1). These results indicate that part of the anticonvulsant action may be different than the action of these mutations. The effects of ethosuximide and/or trimethadione were only partially additive with several

Table 1. Mean and maximum life-spans. Most strains were fed live *E. coli* OP50 and cultured at 20°C. Exceptions were wild-type strain N2 cultured at 15°C (WT, 15°C), N2 fed live *B. subtilis* (WT, *B. subtilis*), and N2 fed UV-killed OP50 (WT, UV/*E. coli*). External drug concentrations are shown in milligrams per milliliter for ethosuximide (ETH), trimethadione (TRI), and succinimide (SUC). (4/0) and (0/4) indicate culture with drug from fertilization to L4 and L4 to death, respectively. Genotypes with no drug treatment are compared with line 1, and differences were not analyzed for statistical significance. Otherwise, comparisons are to the same genotype with no drug treatment. For these comparisons in the columns showing life-spans, numbers with no asterisks are not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$. Maximum adult life-span is the mean life-span of the 10% of the population that had the longest life-spans. *N*, number of hermaphrodites analyzed, with number of independent experiments in parentheses. N.D., not determined.

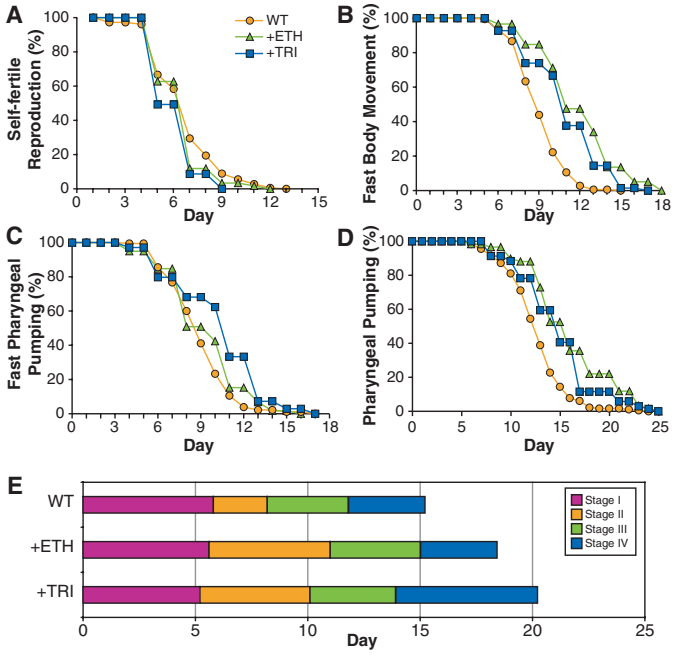
Genotype	Drug	Mean life-span ± SD (days)	% Change in mean life-span	Maximum life-span ± SD (days)	% Change in maximum life-span	*N*
WT	None	16.7 ± 3.7		23.3 ± 1.7		976(19)
	ETH(2)	18.9 ± 6.0***	+13	28.9 ± 1.7***	+24	479(10)
	ETH(4)	19.6 ± 5.3***	+17	28.5 ± 1.8***	+22	458(10)
	TRI(4)	24.6 ± 8.4***	+47	36.5 ± 2.2***	+57	482(9)
	TRI (0/4)	20.7 ± 6.7***	+24	30.1 ± 2.3***	+29	124(2)
	TRI (4/0)	15.5 ± 3.4	–7	21.0 ± 1.0	–10	110(2)
	DEABL(2)	21.8 ± 7.6***	+31	34.7 ± 1.6***	+49	92(2)
	SUC(2)†	16.0 ± 4.0	–4	23.5 ± 1.9	+1	94(2)
WT, 15°C	None	23.6 ± 5.3	+41	32.9 ± 2.7	+41	116(2)
	ETH(4)	31.9 ± 8.2***	+35	45.2 ± 2.4***	+37	93(2)
WT, *B. subtilis*	None	19.5 ± 5.4	+17	28.8 ± 1.5	+24	108(2)
	TRI(4)	27.1 ± 6.6***	+39	38.2 ± 1.8***	+33	115(2)
WT, UV/*E. coli*	None	20.0 ± 6.3	+20	30.4 ± 2.3	+30	51(2)
	ETH(4)	22.8 ± 4.8*	+14	29.8 ± 1.4	–2	45(2)
	TRI(4)	28.1 ± 6.0***	+41	37.3 ± 0.7**	+23	49(2)
daf-16 (m26)	None	14.4 ± 3.3	–14	20.0 ± 1.0	–14	123(3)
	ETH(2)	16.7 ± 3.0***	+16	21.3 ± 0.8**	+7	119(3)
	TRI(2)	16.7 ± 3.2***	+16	22.0 ± 0.0 (N.D.)	+10	58(1)
daf-16 (mu86)	None	14.4 ± 3.2	–14	19.6 ± 0.9	–16	113(2)
	ETH (0.5)	16.0 ± 3.5**	+11	21.1 ± 0.5**	+8	105(2)
	TRI(4)	17.4 ± 4.5**	+21	24.2 ± 1.2**	+23	53(1)
daf-2 (e1370)	None	34.6 ± 10.9	+107	52.6 ± 5.0	+126	142(3)
	ETH(4)	39.3 ± 11.5**	+14	56.8 ± 2.9*	+8	118(3)
	TRI(4)	37.6 ± 8.5*	+9	50.0 ± 3.5	–5	50(1)
unc-31 (e928)	None	22.8 ± 8.4	+37	36.2 ± 3.3	+55	56(3)
	ETH(2)	27.4 ± 11.1**	+20	44.4 ± 2.4***	+23	95(3)
	TRI(4)	28.3 ± 5.4***	+24	35.8 ± 2.4	–1	55(1)
unc-64 (e246)	None	22.6 ± 10.7	+35	42.8 ± 3.3	+84	227(4)
	ETH(2)	24.4 ± 10.7*	+8	43.7 ± 2.6*	+2	160(3)
	TRI(4)	28.1 ± 10.2***	+24	43.3 ± 2.9	+1	245(2)
aex-3 (ad418)	None	19.3 ± 5.3	+16	29.1 ± 2.5	+25	209(4)
	ETH(4)	21.8 ± 6.8***	+13	34.4 ± 2.1***	+18	236(4)
	TRI(4)	26.0 ± 6.0***	+35	34.3 ± 1.0***	+18	113(2)
tax-4 (p678)	None	20.1 ± 6.6	+20	31.4 ± 2.4	+35	144(3)
	TRI(4)	27.4 ± 4.9***	+36	35.2 ± 1.2*	+12	52(1)
osm-3 (p802)	None	20.2 ± 6.6	+21	32.1 ± 4.1	+38	161(4)
	ETH(2)	22.1 ± 7.1	+9	34.0 ± 3.6	+6	131(3)
	TRI(4)	23.6 ± 5.4**	+17	32.7 ± 3.0	+2	30(1)
eat-2 (ad465)	None	20.1 ± 6.3	+20	31.4 ± 3.1	+35	192(4)
	TRI(4)	28.6 ± 10.0***	+42	47.2 ± 7.3**	+50	68(1)

†Concentrations of 0.5, 5, or 10 mg/ml succinimide also did not significantly increase mean life-span.

mutations, notably *daf-2*, *unc-64*, and *osm-3*. Thus, part of the activity of the anticonvulsants may be similar to the effects of these mutations, several of which affect neural function. However, an absence of full additivity is also consistent with other possibilities (*13*).

Anticonvulsants affect the neural activity of vertebrates. To determine whether these drugs have a similar activity in nematodes, we analyzed neuromuscular behaviors. *C. elegans* egg laying is mediated by HSN neurons that innervate the vulval muscles (*16*, *17*). Wild-type hermaphrodites lay eggs that have matured to about the 30-cell stage of development. Trimethadione and ethosuximide caused wild-type hermaphrodites to lay eggs at much earlier stages of development, often the 1- to 7-cell stage (Fig. 3C). The control drug, succinimide, did not stimulate egg laying (Fig. 3C). A delay in egg laying can result in an egg-laying defective (Egl) phenotype characterized by progeny that hatch internally. Approximately 8.9% of wild-type hermaphrodites displayed an Egl phenotype during their lifetime; ethosuximide and trimethadione reduced this to 2.9 and 1.2%, respectively (Fig. 3A). To investigate whether the anticonvulsants act presynaptically on the HSN neurons or postsynaptically on the vulval muscles, we analyzed an *egl-1* mutant that lacks HSNs as a result of a developmental abnormality (*17*). Ethosuximide did not cause *egl-1* mutants to lay eggs at earlier stages of development (Fig. 3D), indicating that the vulval muscles are not sufficient and the HSN neurons are necessary for the anticonvulsant to stimulate egg laying. This result is consistent with the model that the drug acts presynaptically.

Treatment with ethosuximide or trimethadione caused wild-type hermaphrodites to display hyperactive motility, indicating that these drugs stimulate neuromuscular activity (Fig. 3B). To analyze this phenotype, we



F

Drug	Mean Span (Days) Self-fertile reproductive	Fast body movement	Fast pharyngeal pumping	Pharyngeal pumping	N
WT	5.8 ± 2.0	8.2 ± 1.7	8.1 ± 2.1	11.8 ± 3.0	180
+ETH	5.6 ± 1.5	11.0 ± 2.9***	8.4 ± 2.7	15.0 ± 4.2***	59
+TRI	5.2 ± 1.3**	10.1 ± 2.7***	9.4 ± 3.1**	13.9 ± 3.9***	69

Fig. 2. Anticonvulsants delay age-related declines of physiological processes. Wild-type hermaphrodites were cultured with no drug (WT), 2 mg/ml ethosuximide (+ETH), or 4 mg/ml trimethadione (+TRI). We measured the time from L4 to the cessation of self-fertile progeny production (**A**), to the cessation of fast body movement (**B**), to the cessation of fast pharyngeal pumping (≥25 contractions per 10 s) (**C**), and to the cessation of all pharyngeal pumping (≥1 contraction per 10 s) (**D**). (**E**) Stages I to IV end at the mean self-fertile reproductive span, the mean fast body movement span, the mean pharyngeal pumping span, and the mean lifespan, respectively. (**F**) Mean values in days ± SD for data in (A) to (D). Stars indicate *P* values compared with no drug (Table 1).

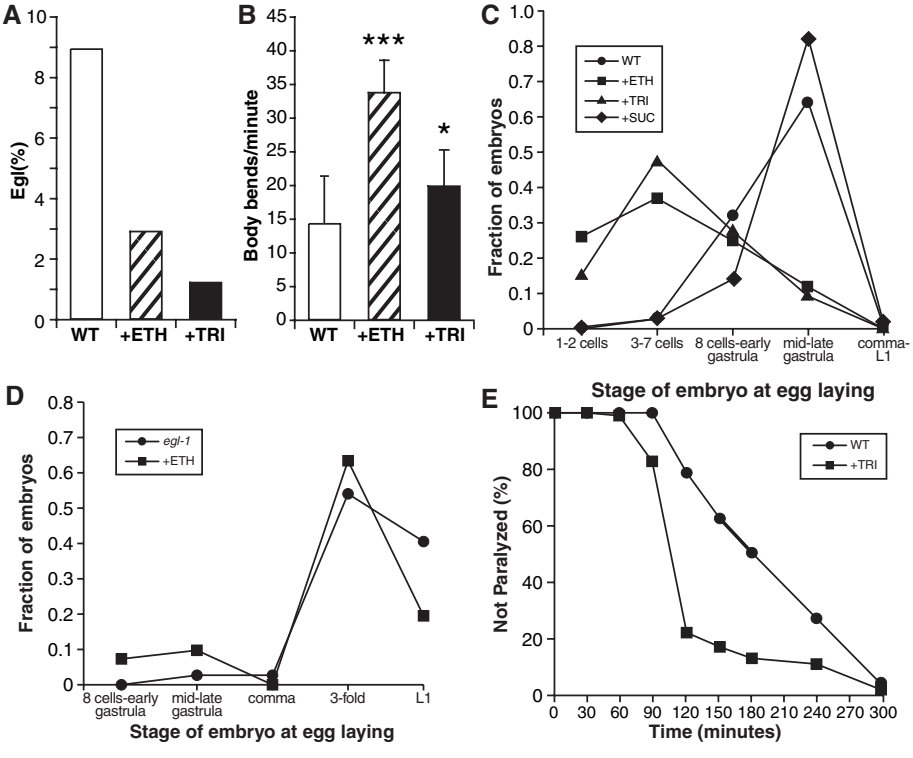


Fig. 3. Anticonvulsants stimulate neuromuscular activity. (**A**) The percent of dead hermaphrodites that displayed internally hatched progeny (Egl) with no drug (WT), 2 mg/ml ethosuximide (+ETH), or 4 mg/ml trimethadione (+TRI) ($n > 150$). (**B**) Motility of wild-type young adult hermaphrodites with no drug ($n = 13$), 2 mg/ml ethosuximide ($n = 8$), 4 mg/ml trimethadione ($n = 14$). Stars indicate *P* values compared to no drug (Table 1). (**C** and **D**) The developmental stage of embryos at the time of egg laying. (C) Wild-type young adult hermaphrodites treated with no drug ($n = 107$), 2 mg/ml ethosuximide ($n = 92$), 4 mg/ml trimethadione ($n = 119$), or 2 mg/ml succinimide (+SUC) ($n = 44$). (D) *egl-1(n487)* young adult hermaphrodites treated with no drug ($n = 37$) or 2 mg/ml ethosuximide ($n = 41$). (**E**) A time course of paralysis induced by aldicarb in wild-type young adult hermaphrodites treated with no drug ($n = 99$) or 4 mg/ml trimethadione ($n = 99$).

examined sensitivity to the acetylcholinesterase inhibitor aldicarb. Aldicarb causes paralysis of body movement resulting from the accumulation of acetylcholine at the neuromuscular junction (*18*). Mutations that reduce synaptic transmission cause resistance to aldicarb (*18*). In contrast, mutations that stimulate synaptic transmission cause hypersensitivity to aldicarb-mediated paralysis (*19*). Trimethadione treatment of wild-type animals caused hypersensitivity to aldicarb-mediated paralysis (Fig. 3E). The control drug, succinimide, did not cause hyperactive motility or aldicarb hypersensitivity (*3*). These results indicate the anticonvulsants stimulate synaptic transmission in the neuromuscular system that controls body movement.

Ethosuximide and trimethadione effectively treat absence seizures in humans by regulating neural activity. A likely target of ethosuximide is T-type calcium channels, although it is possible that these compounds act on multiple targets (*20*–*22*). These anticonvulsants also affected neural activity in nematodes, and the anticonvulsant and the life-span extension effects of the compounds may act through similar mechanisms. The findings presented here are consistent with the model that the effect on neural activity causes the life-span extension, although they do not exclude the possibility that the drugs affect neural activity and aging by different mechanisms. Furthermore, the interactions with the insulin-signaling mutants suggest the intriguing possibility that neural activity regulates aging by both *daf-16*–dependent and *daf-16*–independent mechanisms.

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Materials and Methods
Fig. S1
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Self-Propagating, Molecular-Level Polymorphism in Alzheimer's β-Amyloid Fibrils

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Amyloid fibrils commonly exhibit multiple distinct morphologies in electron microscope and atomic force microscope images, often within a single image field. By using electron microscopy and solid-state nuclear magnetic resonance measurements on fibrils formed by the 40-residue β-amyloid peptide of Alzheimer's disease ($A\beta_{1–40}$), we show that different fibril morphologies have different underlying molecular structures, that the predominant structure can be controlled by subtle variations in fibril growth conditions, and that both morphology and molecular structure are self-propagating when fibrils grow from preformed seeds. Different $A\beta_{1–40}$ fibril morphologies also have significantly different toxicities in neuronal cell cultures. These results have implications for the mechanism of amyloid formation, the phenomenon of strains in prion diseases, the role of amyloid fibrils in amyloid diseases, and the development of amyloid-based nanomaterials.

Amyloid fibrils are self-assembled filamentous aggregates formed by peptides and proteins with diverse amino acid sequences (*1*). Current interest in amyloid fibrils arises from their involvement in Alzheimer's disease (AD), type 2 diabetes, prion diseases, and other protein misfolding disorders (*2*) and from basic questions about the interactions that stabilize amyloid structures and the mechanisms by which they form (*3*). Recent experiments additionally suggest that amyloid structures may be a basis for one-dimensional nanomaterials with possible technological applications (*4*, *5*).

In transmission electron microscope (TEM) and atomic force microscope (AFM) images, amyloid fibrils commonly exhibit multiple distinct morphologies, often described as twisted or parallel assemblies of finer protofilaments (*6*–*8*). Two explanations for amyloid polymorphism are possible: (i) distinct morphologies result from distinct modes of lateral association of protofilaments without significant variations in molecular structure (*7*) or (ii) distinct morphologies result from significant variations in molecular structure at the protofilament level. Here, we report electron microscopy and solid-state nuclear magnetic resonance (NMR) data on amyloid fibrils formed by the 40-residue β-amyloid peptide associated with AD ($A\beta_{1–40}$) that support the second possibility and reveal specific molecular-level structural differences between different fibril morphologies. The predominant morphology and molecular structure are sensitive to subtle differences in fibril growth conditions in de novo preparations (at fixed pH, temperature, buffer composition, and peptide concentration), but both morphology and molecular structure are self-propagating in seeded preparations. Different $A\beta_{1–40}$ fibril morphologies also exhibit significantly different toxicities in neuronal cell cultures.

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Amyloid fibrils were prepared from the human $A\beta_{1-40}$ peptide (sequence NH_2-DAEFRHDSGY EVHHQKLVFF AEDVGSNKGA IIGLMVGGVV-COOH) (*9*, *10*). Parent fibrils were grown by incubation of identical fresh $A\beta_{1-40}$ solutions for periods of 21 to 68 days either in vertical dialysis tubes in an unstirred bath of buffer or in horizontal polypropylene tubes with gentle circular agitation (Red Rotor orbital mixer, Hoefer Scientific Instruments, San Francisco, CA), producing quiescent and agitated parent fibrils, respectively. Daughter fibrils were grown under dialysis conditions by seeding fresh solutions with sonicated fragments (9.1% of total $A\beta_{1-40}$) of either quiescent or agitated parents. Daughter fibrils were used as seeds for granddaughter fibrils, also grown under dialysis conditions. Daughter and granddaughter fibrils were grown for 3 to 8 days.

TEM images of negatively stained $A\beta_{1-40}$ fibrils are shown in Fig. 1 (*9*). The predominant morphology for quiescent parents is a fibril with a periodically modulated width (50- to 200-nm period and 12- ± 1-nm maximum width), commonly ascribed to a periodic twist (*6*–*8*). The predominant morphology for agitated parents is a filament with no resolvable twist (5.5- ± 0.5-nm width) but with a pronounced tendency to associate laterally into dimers or multimers. Morphological differences are preserved in TEM images of sonicated seeds and are transmitted to daughter and granddaughter fibrils, even though all daughter and granddaughter fibrils were grown under identical dialysis conditions. AFM images show similar morphological differences (fig. S1).

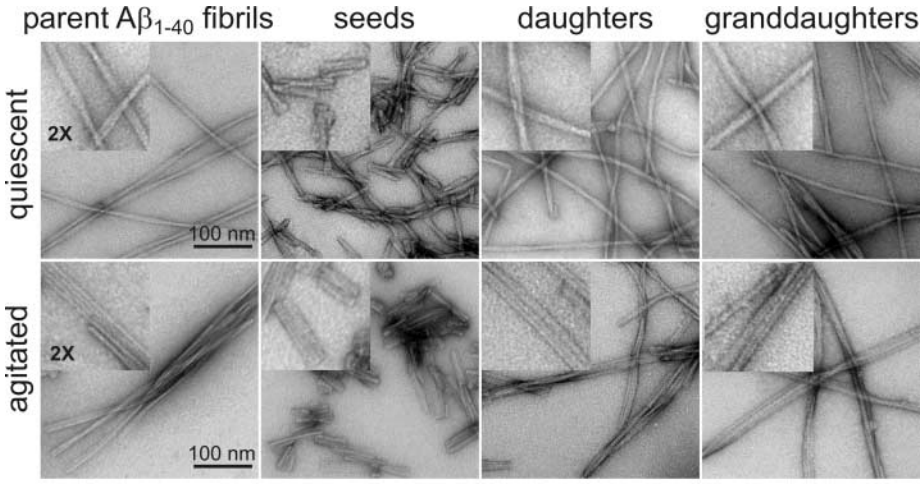

Fig. 1. TEM images of amyloid fibrils formed by the $A\beta_{1-40}$ peptide, negatively stained with uranyl acetate. Parent fibrils were prepared by incubation of $A\beta_{1-40}$ solutions either under quiescent dialysis conditions or in a closed polypropylene tube with gentle agitation. Daughter and granddaughter fibrils were grown under dialysis from solutions that were seeded with sonicated fragments of parent and daughter fibrils, respectively.

Molecular and supramolecular structures of amyloid fibrils can be probed by various solid-state NMR techniques (*3*, *11*–*16*). Figure 2 shows two-dimensional (2D) solid-state ^{13}C NMR spectra of parent, daughter, and granddaughter fibrils with uniform labeling of F20, D23, V24, K28, G29, A30, and I31 (*9*). The patterns of strong one-bond cross peaks in these spectra are determined by isotropic ^{13}C chemical shifts, which are sensitive to local structural and conformational environment (*13*, *17*, *18*). Remarkably, the 2D spectra of quiescent and agitated parent fibrils show pronounced differences in cross-peak patterns that are transmitted to the daughter and granddaughter fibrils. Thus, the morphological differences in Fig. 1 correlate with underlying differences in structure at the molecular level. The ^{13}C chemical shift differences are greater than 1.5 parts per million (ppm) at multiple backbone and side-chain

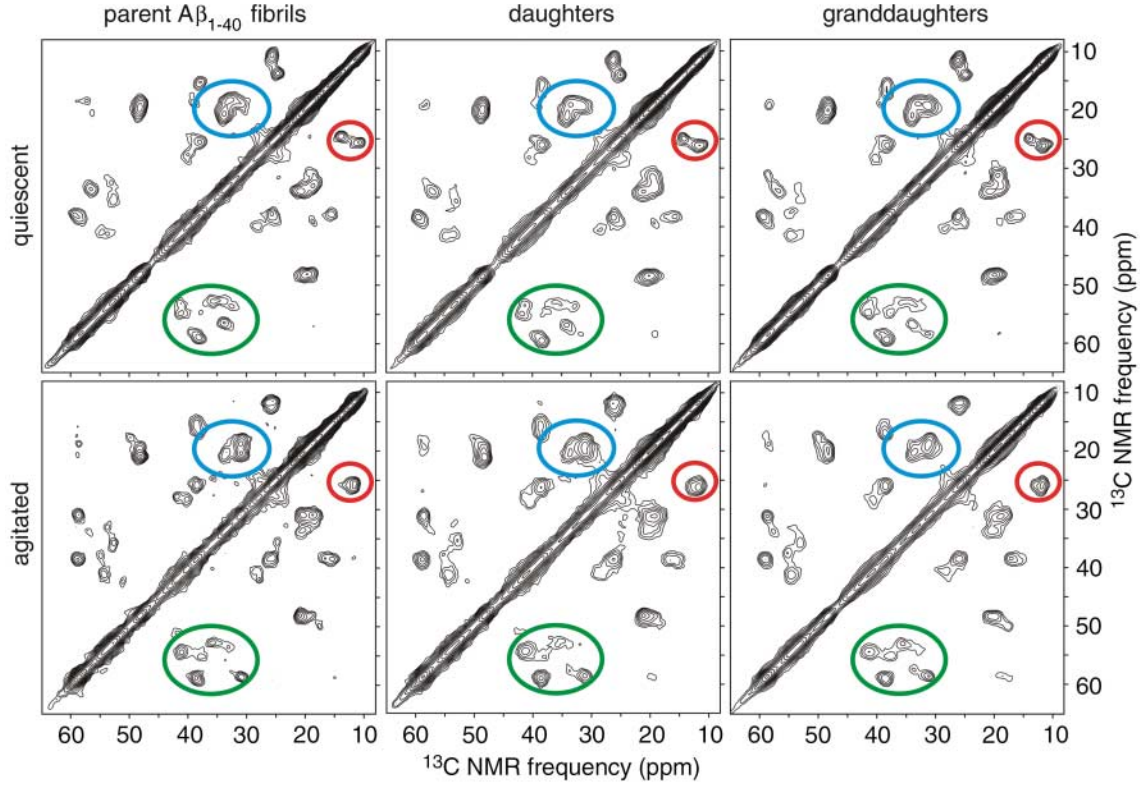

Fig. 2. Two-dimensional solid-state ^{13}C NMR spectra of $A\beta_{1-40}$ fibrils, prepared with ^{13}C labeling of all carbon sites in amino acid residues F20, D23, V24, K28, G29, A30, and I31. Spectra were recorded under magic angle spinning. Regions enclosed by ellipses contain $C_{\gamma 1}/C_{\delta}$ cross peaks of I31 (red), C_{β}/C_{γ} cross peaks of V24 (blue), and C_{α}/C_{β} cross peaks of F20, D23, V24, K28, and I31 (green).

sites (tables S1 and S2) and are independent of the protocol used to prepare $A\beta_{1-40}$ solutions for parent fibril growth (fig. S2).

The propensities for lateral association exhibited by quiescent and agitated fibrils suggest that different amino acid side chains are exposed on the fibril surfaces, possibly leading to different biological activities. Figure 3 shows the results of measurements of $A\beta_{1-40}$ fibril toxicity in cultures of primary rat embryonic hippocampal neurons (*9*). Both quiescent and agitated fibrils are neurotoxic at $A\beta_{1-40}$ concentrations of 10 μM and above, but the toxicity of quiescent fibrils is significantly higher than that of agitated fibrils. Toxicity measurements on nonfibrillar $A\beta_{1-40}$ aggregates, which form in unseeded solutions at early stages of incubation, show no significant difference between quiescent and agitated conditions (fig. S3).

Some specific structural features of quiescent and agitated $A\beta_{1-40}$ fibrils are indicated in Fig. 4. All amyloid structures contain the cross-β motif, i.e., β sheets extending over the length of the fibril, with β strands roughly perpendicular to and interstrand hydrogen bonds roughly parallel to the long fibril axis (*1*, *3*). Intermolecular ^{13}C-^{13}C nuclear magnetic dipole-dipole couplings (Fig. 4A) indicate intermolecular distances of 0.55 ± 0.05 nm at V12, V39, and A30, implying in-register, parallel β sheets for both morphologies as reported previously for $A\beta_{10-35}$ and $A\beta_{1-40}$ fibrils prepared with different protocols (*12*, *14*, *15*, *19*). The ^{15}N-^{13}C dipole-dipole couplings (Fig. 4B) indicate a 0.32 ± 0.02-nm distance between side-chain C_γ carbons of D23 residues and side-chain N_ζ nitrogens of K28 residues in agitated $A\beta_{1-40}$ fibrils, consistent with salt bridges between oppositely charged D23 and K28 side chains. Weaker D23-K28 couplings are observed in quiescent fibrils, consistent with longer average C_γ-N_ζ distances. Dipole-dipole couplings between side-chain C_δ carbons of E22 residues and side-chain N_ζ nitrogens of K16 residues are observed in quiescent fibrils, possibly indicating partial occupation of an intermolecular K16-E22 salt bridge, but are absent in agitated fibrils. Histograms of fibril mass-per-length (MPL) (Fig. 4C) obtained from scanning transmission electron microscope (STEM) images (*9*) show mode values of 21.4 kD/nm for agitated fibrils [similar to earlier $A\beta_{1-40}$ and $A\beta_{1-42}$ fibril STEM data (*6*, *15*)] and 30.3 kD/nm for quiescent fibrils. Given the 0.47- to 0.48-nm spacing between peptide chains in an ideal cross-β structure, the MPL of one layer of $A\beta_{1-40}$ molecules (4.3 kD mass) would be 9.1 kD/nm. Figure 4C suggests that the protofilament (defined here as the experimentally detected structure with minimal MPL) contains two molecular layers in agitated $A\beta_{1-40}$ fibrils and three molecular layers in quiescent $A\beta_{1-40}$ fibrils. Deviations from precise integer multiples of 9.1 kD/nm may arise from a nonzero angle θ between the interstrand hydrogen bonding direction and the long fibril axis, which would increase the MPL values by 1/cos θ.

Established correlations between ^{13}C NMR chemical shifts and secondary structure (*13*, *17*) and predictions of backbone ϕ and ψ torsion angles from the TALOS program (*18*) suggest that the β strand segments in quiescent $A\beta_{1-40}$ fibrils include residues 10 to 14, 16 to 22, 30 to 32, and 34 to 36, whereas those in agitated $A\beta_{1-40}$ fibrils include residues 10 to 22, 30 to 32, and 34 to 36 (tables S1 and S2). Figure 4A shows that V12 and V39 participate in the parallel, hydrogen-bonded structure in both polymorphs. The ^{13}C chemical shifts for Q15, D23, G25, and G33 in quiescent fibrils and for D23, G25, and G33 in agitated fibrils suggest non-β strand conformations at these residues. The ^{13}C NMR linewidths (fig. S4) indicate structurally disordered N-terminal segments in both morphologies, as previously suggested (*13*, *14*, *19*–*21*). Linewidths for most CO, C_α, and C_β sites in residues 12 to 39 of quiescent fibrils and residues 10 to 39 of agitated fibrils are 2.5 ppm or less, indicating overall structural order (*13*).

Data for agitated $A\beta_{1-40}$ fibrils are largely consistent with our recent model for the

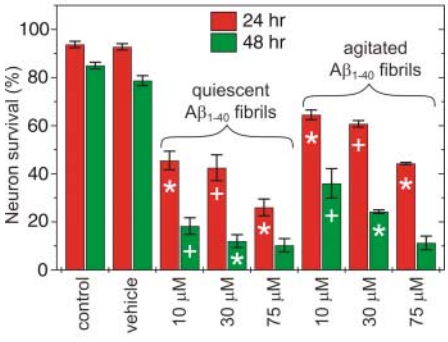

Fig. 3. Toxicity of $A\beta_{1-40}$ fibrils in cultures of primary embryonic rat hippocampal neurons, assayed by counting viable neurons after 24-hour and 48-hour exposures to $A\beta_{1-40}$ at indicated concentrations in neurobasal medium. Error bars indicate standard errors on mean survival values (four culture dishes per condition and 35 to 145 neurons monitored per dish). Control is neurobasal medium alone. Vehicle is supernatant after centrifugation of $A\beta_{1-40}$ fibril solution, added to neurobasal medium in volume equal to that for 30 μM conditions. The asterisks and plus symbols indicate conditions where toxicity differences between quiescent and agitated fibrils are statistically significant (analysis of variance test, $P < 0.01$ and $P < 0.05$, respectively).

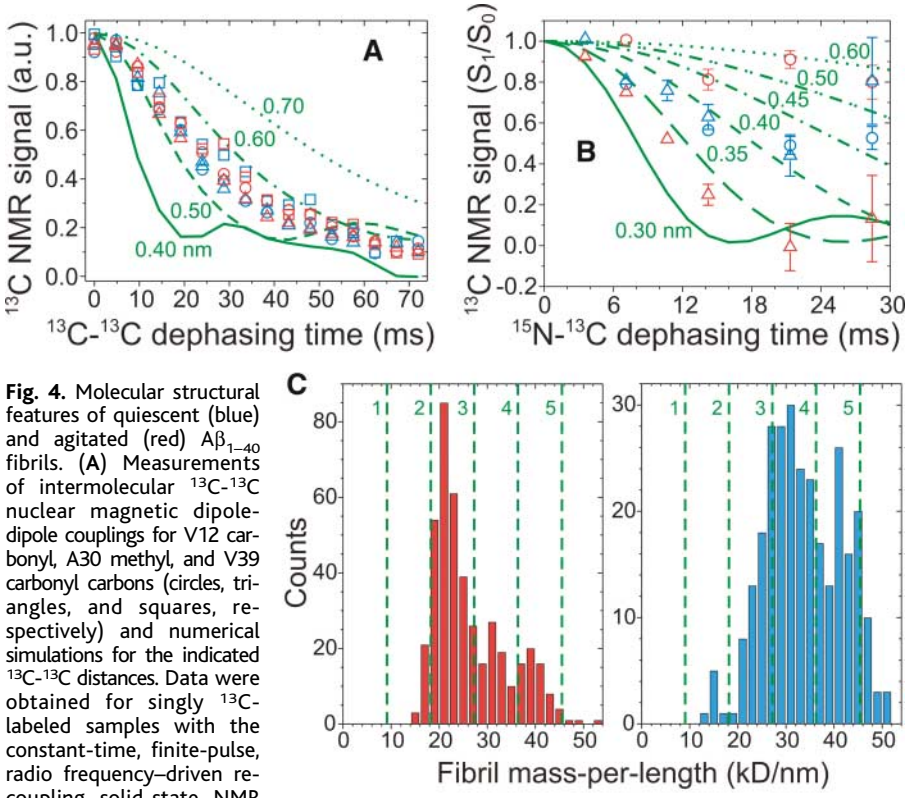

Fig. 4. Molecular structural features of quiescent (blue) and agitated (red) $A\beta_{1-40}$ fibrils. (**A**) Measurements of intermolecular ^{13}C-^{13}C nuclear magnetic dipole-dipole couplings for V12 carbonyl, A30 methyl, and V39 carbonyl carbons (circles, triangles, and squares, respectively) and numerical simulations for the indicated ^{13}C-^{13}C distances. Data were obtained for singly ^{13}C-labeled samples with the constant-time, finite-pulse, radio frequency–driven recoupling solid-state NMR technique (*9*, *14*). (**B**) Measurements of ^{15}N-^{13}C dipole-dipole couplings for D23 C_γ/K28 N_ζ and E22 C_δ/K16 N_ζ pairs (triangles and circles, respectively) and numerical simulations for the indicated ^{15}N-^{13}C distances. Data were obtained for samples with uniformly ^{15}N- and ^{13}C-labeled residues with the frequency-selective rotational echo double resonance solid-state NMR technique (*9*, *32*). Error bars are calculated from the root mean square noise in the NMR spectra. (**C**) MPL histograms extracted from STEM images of $A\beta_{1-40}$ fibrils. Dashed lines indicate MPL values for ideal cross-β structures with between one and five layers of $A\beta_{1-40}$ molecules. a.u., arbitrary units.

molecular structure of the $A\beta_{1-40}$ protofilament (*13*, *15*). STEM, ^{15}N-^{13}C coupling, and chemical shift data for quiescent $A\beta_{1-40}$ fibrils indicate a qualitatively different structure in both molecular conformation and supramolecular organization. The largest chemical shift differences, suggesting the largest conformational differences, occur at Q15 and in residues 22 to 28. Although all residues in agitated $A\beta_{1-40}$ fibril samples exhibit one major set of ^{13}C chemical shifts (with the exception of D23, V24, and K28, possibly indicating the coexistence of two distinct D23-K28 salt bridge geometries), many residues in quiescent fibril samples exhibit two sets of ^{13}C chemical shifts, with an approximate 2:1 ratio of NMR signal intensities (e.g., I31 side-chain signals in Fig. 2). This observation raises the possibility that the quiescent $A\beta_{1-40}$ protofilament contains two structurally equivalent and one structurally inequivalent subunits, consistent with Fig. 4, B and C.

We point out four physical and biological implications of these data. First, the sensitivity of fibril morphology and molecular structure to growth conditions shows that at least two distinct fibril nucleation mechanisms exist for $A\beta_{1-40}$. One mechanism, leading to quiescent fibrils, may be purely homogeneous. The other mechanism, leading to agitated fibrils, may depend on the interface between the peptide solution and the air or the walls of the sample tube. The molecular structure of $A\beta_{1-40}$ fibrils is not determined solely by amino acid sequence and is not purely under thermodynamic control. Second, the phenomenon of strains in prion diseases, in which a single prion protein gives rise to multiple, distinct phenotypes, has been attributed to an ability of both mammalian and yeast prion proteins to adopt multiple, distinct amyloid-like structures. Observed differences in proteolysis patterns (*22*, *23*), resistance to chemical denaturation (*24*), seeding efficiencies (*25*), and electron paramagnetic resonance signals (*26*) support this proposal, but clear connections between morphological variations and molecular-level structural variations, between strains and morphological variations, and between strains and specific features of molecular structure have not yet been established experimentally. The correlations of amyloid fibril morphology with specific structural features established by our data, the demonstration of their self-propagating nature, and the observation of different neurotoxicities for different morphologies further strengthen the case for a structural origin of prion strains. Third, the importance of mature amyloid fibrils as etiological agents in AD and other amyloid diseases, as opposed to nonfibrillar oligomers observed at earlier stages of peptide incubation (*27*–*30*), is a subject of current controversy. One principal argument against a primary role for mature fibrils in AD has been the absence of a robust correlation between the severity of neurological impairment and the extent of amyloid deposition (*2*, *31*). Data in Fig. 3 raise the possibility that certain amyloid morphologies may be more pathogenic than others in the affected organs of amyloid diseases, which would weaken the correlation between disease symptoms and total amyloid deposition. Fourth, amyloid fibrils may prove useful as structural and chemical templates for self-assembled, one-dimensional nanomaterials with novel electronic or optical properties (*4*, *5*). Because structural uniformity is a likely prerequisite in such applications, the self-propagation of molecular structure demonstrated above may be important for reliable fabrication of amyloid-based nanomaterials.

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9. Materials and methods are available as supporting material on *Science* Online.
10. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supporting Online Material

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Materials and Methods
Figs. S1 to S4
Tables S1 and S2

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Semaphorin 3E and Plexin-D1 Control Vascular Pattern Independently of Neuropilins

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The development of a patterned vasculature is essential for normal organogenesis. We found that signaling by semaphorin 3E (Sema3E) and its receptor plexin-D1 controls endothelial cell positioning and the patterning of the developing vasculature in the mouse. Sema3E is highly expressed in developing somites, where it acts as a repulsive cue for plexin-D1–expressing endothelial cells of adjacent intersomitic vessels. Sema3E–plexin-D1 signaling did not require neuropilins, which were previously presumed to be obligate Sema3 coreceptors. Moreover, genetic ablation of Sema3E or plexin-D1 but not neuropilin-mediated Sema3 signaling disrupted vascular patterning. These findings reveal an unexpected semaphorin signaling pathway and define a mechanism for controlling vascular patterning.

The peripheral nervous system and its vasculature develop coordinately in part through common developmental cues. Semaphorins, a family of phylogenetically conserved cell-surface and secreted proteins, control neuronal cell migration and axon guidance (*1*–*3*). Certain membrane-bound semaphorins bind directly to receptors of the plexin family (*4*–*7*), but the class 3 secreted semaphorins (Sema3A to Sema3F, referred to collective-

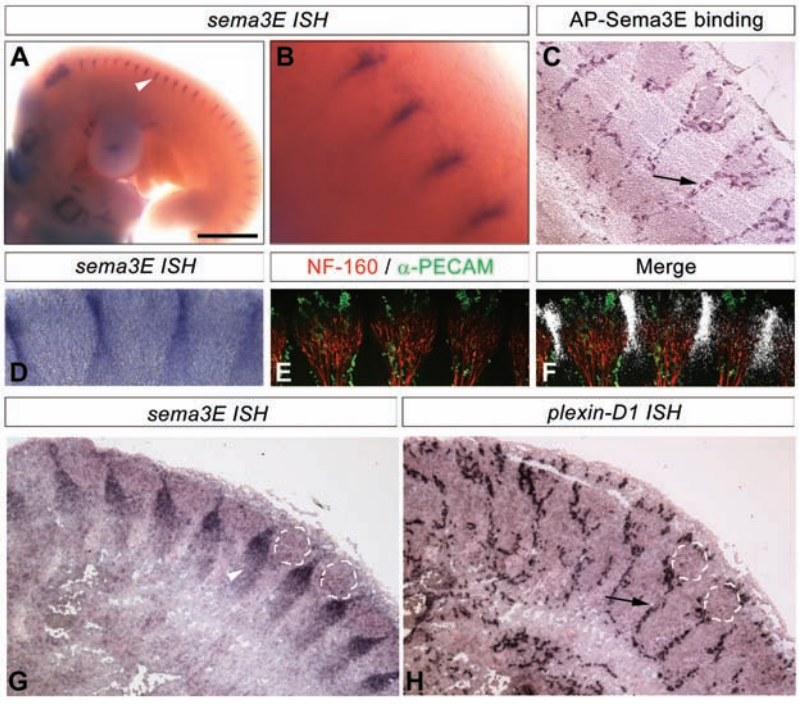

Fig. 1. Complementary expression patterns of *sema3E* in somites and *plexin-D1* and Sema3E binding sites on intersomitic blood vessels. (**A**) Whole-mount in situ hybridization (ISH) was performed on E11.5 embryos to visualize the pattern of expression of *sema3E*. In the trunk region, the highest level of Sema3E mRNA was detected in the caudal region of each somite. White arrowhead, region of *sema3E* mRNA expression. (**B**) High magnification of *sema3E* in situ hybridization shown in (A). (**C**) AP-Sema3E section binding to sagittal sections from E11.5 embryos. Black arrow, intersomitic vessels. (**D**) *Sema3E* in situ hybridization of a 100-μm-thick sagittal section of the E11.5 embryo shown in (A). (**E**) The embryo shown in (D) was immunostained with anti–platelet endothelial cell adhesion molecule (PECAM) (green) to visualize the vasculature and antineurofilament (red) to visualize spinal nerves and dorsal root ganglia (DRG). (**F**) Overlay of (D) and (E); *sema3E* mRNA [inversion of (D)], white; neurofilament, red; PECAM, green. (**G**) *Sema3E* in situ hybridization of an E11.5 embryo sagittal section. (**H**) *Plexin-D1* in situ hybridization of an E11.5 embryo sagittal section. White dotted lines outline the DRG. Scale bar, 1.2 mm (A); 300 μm [(B), (C), (G), and (H)]; 150 μm [(D) to (F)].

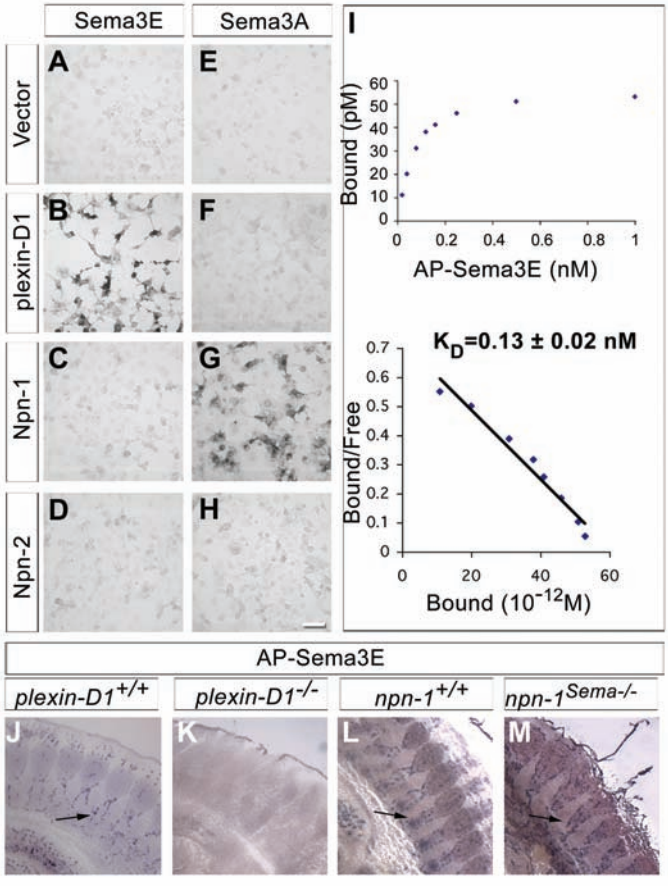

Fig. 2. Sema3E but not Sema3A binds with high affinity to plexin-D1, the endogenous receptor for Sema3E. (**A** to **D**) AP-Sema3E binds to COS-1 cells expressing plexin-D1 but not Npn-1 or Npn-2. (**E** to **H**) AP-Sema3A binds to COS-1 cells expressing Npn-1 but not plexin-D1 or Npn-2. COS-1 cells were transfected with either an empty vector [(A) and (E)] or an expression vector encoding plexin-D1 [(B) and (F)], Npn-1 [(C) and (G)], or Npn-2 [(D) and (H)]. Cells were incubated with either AP-Sema3E (0.5 nM) or AP-Sema3A (0.5 nM). (**I**) AP-Sema3E binding analysis reveals a tight association between AP-Sema3E and plexin-D1. The binding data were plotted by the method of Scatchard. The apparent K_D (±SEM) for the interaction between AP-Sema3E and plexin-D1 is 0.13 ± 0.02 nM (n = 8). (**J** and **K**) AP-Sema3E binds robustly to the vasculature in sections from wild-type (J) but not *plexin-D1*$^{-/-}$ (K) embryos. (**L** and **M**) Npn-1 is not an endogenous binding partner for Sema3E. AP-Sema3E binding to tissue sections prepared from E11.5 *npn-1*$^{Sema-/-}$ and wild-type littermate embryos. Black arrows, intersomitic vessels. Scale bar, 50 μm [(A) to (H)]; 60 μm [(J) to (M)].

ly as Sema3s) are thought to exert their effects exclusively through holoreceptor complexes that include neuropilin-1 (Npn-1) or Npn-2 and one of the four class A plexin proteins (*2*). Neuropilins function as the Sema3-binding subunits, whereas plexins serve as signal-transducing subunits. Neuropilins are also receptors for select isoforms of vascular endothelial growth factor (VEGF) family members (*8*–*12*). Thus, the severe cardiovascular defects observed in *npn-1* null mice (*13*) and the vascular defects resulting from overexpression of nonselective dominant-negative Npn's (*8*) could reflect a requirement for VEGF–Npn-1 signaling or Sema3–Npn-1 signaling. In vascular development, it is unclear whether Sema3s influence endothelial cell migration by binding to neuropilins, by antagonizing VEGF binding to neuropilins, or by acting through other signaling pathways.

To assess the contributions of Sema3s to vascular development, we focused on Sema3E because it is expressed in developing somites in the mouse (*14*) (Fig. 1, A, B, and G). We

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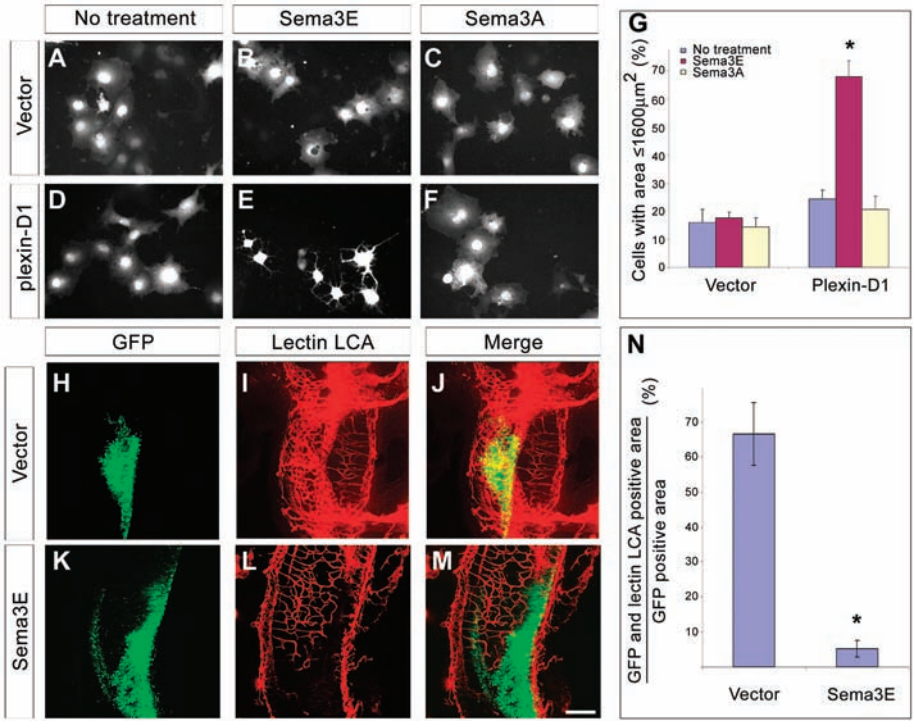

Fig. 3. Sema3E causes collapse of plexin-D1–expressing COS-7 cells and is a repellent for developing chick vasculature. (**A** to **F**) Plexin-D1–transfected COS-7 cells were incubated either with vehicle, Sema3E (AP-Sema3E; 0.1 to 0.5 nM), or Sema3A (AP-Sema3A; 1 to 2 nM). (**G**) COS-7 cell collapse was scored for 200 EGFP-positive cells for each experimental condition. A cell with a surface area less than 1600 μm^2 was considered collapsed (*17*). Shown are the means ± SEM (n = 5). Asterisk indicates statistical difference from all other groups ($P < 0.005$; analysis of variance with a Bonferroni post-hoc test). (**H** to **M**) An expression vector encoding EGFP (GFP) and either an empty vector [Vector, (H) to (J)] or a vector encoding Sema3E [(K) to (M)] were electroporated in ovo into E3 chicken embryos. Two days later, embryos were perfused with Lectin LCA, which binds to the vasculature. Lectin LCA (red) and EGFP (green) fluorescent signals were visualized by confocal microscopy. [(J) and (M)] Merges of Lectin LCA and EGFP signals. (**N**) Quantification of mean overlap (±SEM) between EGFP and Lectin LCA fluorescent signals in vector control and Sema3E electroporated embryos. EGFP alone, n = 7; EGFP + Sema3E, n = 9. Asterisk indicates statistical difference ($P < 0.001$, paired t test). Scale bar, 20 μm [(A) to (F)]; 200 μm [(H) to (M)].

compared the pattern of *sema3E* expression, assessed by in situ hybridization, with the pattern of endogenous Sema3E-binding partners, determined by binding of a chimeric alkaline phosphatase (AP)–Sema3E in embryonic tissues. In mouse embryos at embryonic day 10.5 (E10.5) and E11.5, *sema3E* expression was observed in the caudal region of each somite, immediately adjacent to the somite boundary and intersomitic blood vessels (Fig. 1, D to G, and fig. S1). In contrast, *sema3E* mRNA was not observed within the region of the somite containing the intersomitic blood vessels themselves (Fig. 1, D to G). However, AP-Sema3E binding sites were detected on the vasculature including the intersomitic vessels (Fig. 1C). Therefore, the expression of Sema3E and its endogenous receptor(s) suggests a role for Sema3E in patterning intersomitic vasculature.

The pattern of AP-Sema3E binding was markedly similar to the expression pattern of a plexin family member, plexin-D1, which localizes to the vasculature in mice (*9*) (Fig. 1H) and is critical for vascular development in both zebrafish and mice (*10*, *11*). In the trunk region of E10.5 and E11.5 mouse embryos, *plexin-D1* was expressed in intersomitic vessels adjacent to the caudal region of somites (Fig. 1, G and H, and fig. S1). To determine whether Sema3E binds to plexin-D1, AP-Sema3E was incubated with transfected COS cells (a monkey kidney cell line) expressing plexin-D1, Npn-1, or Npn-2. AP-Sema3E bound directly to plexin-D1 but to neither Npn-1 nor Npn-2 (Fig. 2, A to D). Moreover, Scatchard analysis revealed that the Sema3E–plexin-D1 interaction occurred at high affinity, with a dissociation constant (K_D) of 130 pM (Fig. 2I). Coexpression of Npn-1 or Npn-2 did not enhance the interaction between Sema3E and plexin-D1 (*15*). None of the other five Sema3s bound to plexin-D1, although four of those proteins (Sema3A, Sema3C, Sema3D, and Sema3F) bound directly to Npn-1, Npn-2, or both Npn's (Fig. 2, E to H, and fig. S2) (*15*). Sema3B at a concentration of 1.1 nM did not bind to either Npn-1 or Npn-2 (*15*).

To determine whether plexin-D1 is an endogenous receptor that mediates Sema3E signaling and blood vessel development, we analyzed *plexin-D1* null mice (fig. S3). We observed little or no AP-Sema3E binding to sections prepared from *plexin-D1* null embryos (Fig. 2, J and K), indicating that plexin-D1 is essential for Sema3E binding. In contrast, we observed essentially identical AP-Sema3E binding to tissue sections prepared from wild-type embryos and embryos expressing a Npn-1 variant that binds to VEGF isoforms but not class 3 semaphorins [*npn-1*$^{Sema-/-}$ (*16*)] (Fig. 2, L and M). AP-Sema3A binding sites were, however, undetectable in sections prepared from *npn-1*$^{Sema-/-}$ mice (*15*, *16*). AP-Sema3E also bound to sections prepared from *npn-2*$^{-/-}$ embryos (*15*). The binding of Sema3E to plexin-D1 indicates that a Sema3 can associate directly with a plexin, independently of a neuropilin. We used a cell collapse assay (*17*) to assess whether Sema3E can signal through plexin-D1 in a neuropilin-independent manner. Treatment of plexin-D1–expressing COS-7 cells with Sema3E but not Sema3A induced collapse of lamellipodia (Fig. 3). Together, these findings indicate that plexin-D1 is the endogenous high-affinity receptor for Sema3E and that this ligand-receptor pair mediates cytoskeletal signaling and cell contraction independently of neuropilins.

We next determined whether ectopic expression of Sema3E affects blood vessel patterning in vivo. Sema3E was overexpressed in chick embryos by in ovo electroporation of expression vectors encoding Sema3E and green fluorescent protein (EGFP) into the somites. Embryonic vasculature was visualized after 48 hours with a rhodamine-conjugated lectin (LCA) solution (*18*). Few, if any, vessels were observed in areas of ectopic Sema3E expression (Fig. 3, K to N), whereas vascular patterning appeared normal in regions outside sites of ectopic Sema3E expression (Fig. 3, K to M). Electroporation of EGFP and the empty expression vector had no effect on vascular patterning or somite cytoarchitecture (Fig. 3, H to J, N). These experiments support the view that Sema3E expressed by somites acts as a repulsive cue to restrict vessel growth and branching to intersomitic regions during embryogenesis.

To address whether Sema3E is required in vivo for intersomitic vascular development, we analyzed *Sema3E* null mice (fig. S4). At E11.5, wild-type littermates displayed an iterative pattern of intersomitic blood vessels between each somite. In *Sema3E*$^{-/-}$ mutant embryos, however, intersomitic vessels were disorganized (Fig. 4, A to B′); vessels extended ectopically throughout somites, resulting in exuberant growth and a loss of the normal segmented pattern (n = 14

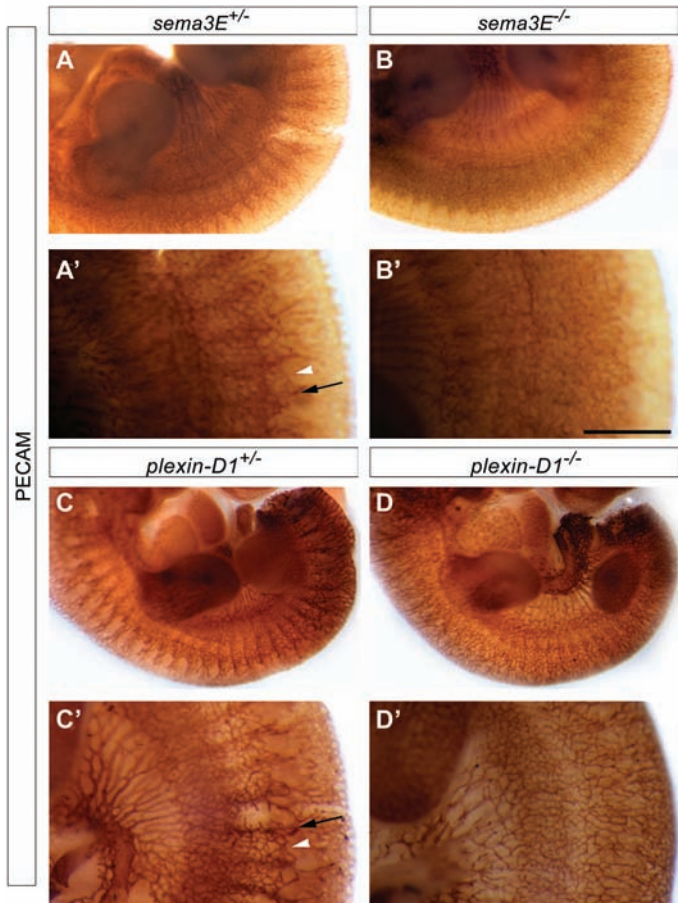

Fig. 4. Sema3E and Plexin-D1 are both required for intersomitic vascular patterning. (**A** and **B**) Whole-mount PECAM staining of E11.5 *sema3E*$^{+/-}$ (A) and *sema3E*$^{-/-}$ (B) mutant embryos showing a Sema3E requirement for normal organization of somitic vasculature. (**A′** and **B′**) High magnification views of E11.5 *sema3E*$^{+/-}$ (A′) and *sema3E*$^{-/-}$ (B′) embryos. The vascular phenotype was observed in all *sema3E*$^{-/-}$ mice (n = 14) but not in heterozygous (n = 4) or wild-type (n = 14) littermates. (**C** and **D**) PECAM staining of E11.5 control (C) and *plexin-D1*$^{-/-}$ mutant (D) embryos showing marked disorganization of somitic vasculature in the absence of plexin-D1 embryos, similar to *sema3E*$^{-/-}$ embryos. (**C′** and **D′**) High-magnification views of E11.5 control (C′) and *plexin-D1*$^{-/-}$ mutant (D′) embryos. The vascular phenotype was observed in all *plexin-D1*$^{-/-}$ mice (n = 8) but not in heterozygous (n = 8) or wild-type littermates (n = 8). White arrowhead, caudal somite; black arrow, intersomitic vessels. Scale bar, 1.2 mm [(A), (B), (C), and (D)]; 0.6 mm [(A′), (B′), (C′), and (D′)].

Sema3E mutants and 14 littermate controls). This phenotype was evident in mice bred into both CD1 and C57BL/6 genetic backgrounds (*15*) and is markedly similar to that observed in mice lacking plexin-D1 (Fig. 4, C to D′, and fig. S6). In *plexin-D1*$^{-/-}$ mice, the iterative pattern of somatic vascular organization was also abolished and vessels extended throughout each entire somite at both E10.5 and E11.5 (n = 8 E11.5 *plexin-D1*$^{-/-}$ mutants and 8 control littermates and n = 4 E10.5 *plexin-D1* mutants and 4 control littermates). This phenotype is comparable to that recently reported in another line of *plexin-D1*$^{-/-}$ mice (*10*) and in plexin-D1–deficient zebrafish (*11*).

The similarity in the nature and extent of vascular defects in *sema3E* and *plexin-D1* mutant mice suggests that somite-derived Sema3E serves as the ligand for plexin-D1 on endothelial cells in vivo. In zebrafish, vascular patterning has been suggested to result from signaling by Sema3A by means of a Npn-1/plexin-D1 complex. Nevertheless, knockdown of Sema3a1 or Sema3a2 resulted only in minor vascular defects that do not recapitulate the *plexin-D1* mutant phenotype (*11*). Conversely, mouse embryos lacking Sema3A show a modest decrease in intersomitic vessel branching (*8*) but, notably, this phenotype is incompletely penetrant and is not observed in all genetic backgrounds (*8*). To assess the in vivo contribution of neuropilins to Sema3E–plexin-D1 signaling, we generated *npn-1*$^{Sema-/-}$;*npn-2* double-mutant mice, in which the interactions between Sema3s and neuropilins are abolished (*15*–*19*). No vascular defect other than persistent truncus arteriosus (*16*) was observed in these mutant embryos (figs. S5 and S7) (*15*). Importantly, there was no difference in intersomitic vasculature between E10.5 and E11.5 *npn-1*$^{Sema-/-}$;*npn-2* double-mutant mice and control littermates (figs. S5 and S7). This finding further supports the view that Sema3E–plexin-D1 signaling is responsible for patterning intersomitic vasculature independently of neuropilins.

The discovery of an unanticipated neuropilin-independent Sema3 signaling pathway adds to the diversity of how Sema3s orchestrate tissue morphogenesis. A complementary pattern of expression of *Sema3E* and *plexin-D1* is also found in other regions of the developing embryo, most prominently the E13.5 forelimb and hindlimb digits, sites where vascular patterning defects are observed in *plexin-D1* mutants (*15*). Thus, Sema3E–plexin-D1 signaling may play a more general role in vascular patterning. Finally, both *Sema3E* and *plexin-D1* are expressed at many specific sites in the nervous system (*9*, *14*, *15*, *20*), and there is a strong association between the development of intersomitic blood vessels and spinal nerves (*21*). Thus, Sema3E–plexin-D1 signaling could also control aspects of neural development.

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Figs. S1 to S7
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Stat3 Dimerization Regulated by Reversible Acetylation of a Single Lysine Residue

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Upon cytokine treatment, members of the signal transducers and activators of transcription (STAT) family of proteins are phosphorylated on tyrosine and serine sites within the carboxyl-terminal region in cells. We show that in response to cytokine treatment, Stat3 is also acetylated on a single lysine residue, Lys[685]. Histone acetyltransferase p300–mediated Stat3 acetylation on Lys[685] was reversible by type I histone deacetylase (HDAC). Use of a prostate cancer cell line (PC3) that lacks Stat3 and PC3 cells expressing wild-type Stat3 or a Stat3 mutant containing a Lys[685]-to-Arg substitution revealed that Lys[685] acetylation was critical for Stat3 to form stable dimers required for cytokine-stimulated DNA binding and transcriptional regulation, to enhance transcription of cell growth–related genes, and to promote cell cycle progression in response to treatment with oncostatin M.

The STAT proteins are latent self-signaling transcription factors in cytoplasm used by most cytokine receptors to rapidly turn on gene expression in nuclei. The regions of STAT that are upstream from the linker to the C terminus appear to participate in protein-protein interactions. This region includes the Src homology 2 (SH2) domain, a short segment (about 30 to 40 amino acids in length) of unknown function, and the transcriptional activation domain (TAD) required for cytokine-induced transcriptional activation. Function-related posttranslational modifications of STAT proteins in response to treatment with cytokine or growth factor include phosphorylation of a single tyrosine residue in the short segment and a single serine residue in the TAD. The SH2 domain of STAT mediates STAT docking on phosphotyrosine (pTyr) motifs of cytokine or growth factor receptors (*1*), and a role in STAT dimerization via reciprocal pTyr-SH2 domain interaction has been also proposed (*2–4*). However, unphosphorylated or tyrosine-mutated STAT proteins can still form dimers and induce transcription (*5–8*), suggesting that another type of regulation contributes to the formation of stable STAT dimers. The C-terminal region of STAT interacts with other proteins during signaling or transcription. For instance, members of the CREB-binding protein (CBP)/p300 family have intrinsic histone acetyltransferase (HAT) activity, associate with various STAT family members within both the C-terminal TAD and N-terminal domain, and increase STAT activity in transcription (*9–12*).

To explore whether STAT proteins themselves are acetylated, we examined Stat3 activation by oncostatin M (OSM), a member of the interleukin-6 (IL-6) cytokine family, and by interferon-α (IFN-α), a type I interferon. In MCF-7 breast cancer cells, OSM treatment induced Stat3 acetylation within 15 min, an event that was maintained for hours (Fig. 1A). Likewise, Stat3 was also acetylated in response to IFN-α treatment in HeLa cells (Fig. 1A). We further examined the location(s) of Stat3 acetylation by separating cytoplasmic and nuclear fractions of MCF-7 cells. OSM-mediated Stat3 phosphorylation and acetylation were detected primarily in the cytoplasmic fraction (fig. S1), consistent with detection of p300 in the cytoplasm of some cell types (*13*). To determine whether p300 might mediate Stat3 acetylation, we transfected cMyc-tagged Stat3 with hemagglutinin (HA)–tagged p300, CBP, or p300/CBP-associated factor (PCAF). In 293T cells, transient transfection of either p300 or CBP increased Stat3 acetylation appreciably, whereas PCAF transfection caused only a small increase in Stat3 acetylation (Fig. 1B).

We evaluated the effect of phosphorylation on Stat3 acetylation by comparing the response of Stat3^{Y705F}, Stat3^{S727A}, and Stat3^{R585Q} (a Stat3 mutant in which Arg^{585} in the SH2 domain was substituted with glutamine) to OSM with the response of wild-type Stat3 in PC3 cells. All three Stat3 mutants were acetylated in cells treated with OSM (*14*). Similarly, transient transfection of 293T cells with p300 caused acetylation of these Stat3 variants (*14*), suggesting that Stat3 phosphorylation and the SH2 domain activity are not prerequisites for Stat3 acetylation. Treatment of 293T cells with trichostatin A (TSA), a broad inhibitor of histone deacetylases (HDACs), further augmented Stat3 acetylation in response to p300 transfection or IFN-α treatment (Fig. 1C), suggesting a role of HDAC factors in Stat3 deacetylation.

Type I HDAC factors may regulate transcription activation by STATs (*15*). To determine whether type I HDAC factors contribute to Stat3 deacetylation, we transfected Hdac1, Hdac2, and Hdac3 with p300 into 293T cells. Stat3 acetylation mediated by p300 was attenuated by Hdac1 and Hdac2 and was almost completely blocked by Hdac3 (Fig. 1D). TSA treatment blocked the negative effect of type-I HDAC on Stat3 acetylation (Fig. 1D). In an in vitro assay, immunoprecipitated acetyl-Stat3 with immunopurified type I HDAC converted Stat3 from its acetylated form into the deacetylated form (Fig. 1E). STAT-dependent transcription can be increased by p300 or CBP transfection (*9*, *10*). To correlate acetylation or deacetylation with Stat3 transcriptional activity, we transfected cells with p300 and HDAC and measured Stat3-dependent luciferase activity. Stat3 was activated in cells transfected with an activated mutant of the tyrosine kinase RET. Stat3 activation was further enhanced in cells transfected with p300 but was inhibited if cells were transfected with type I HDACs. The effect of these HDAC factors was abolished in cells treated with TSA (Fig. 1F). Decreased expression of p300 by up to 90% by small interfering RNA (siRNA) attenuated Stat3 transcriptional activity in response to IL-6 treatment (Fig. 1G). These results indicate that p300 and type I HDAC family members that are present in most cell types may modulate cytokine-induced Stat3 acetylation and deacetylation.

In both MCF-7 and HeLa cells, we detected a complex formation between p300 and Stat3, which was stabilized by treatment with cytokine (Fig. 2A) (*9–11*). We explored Stat3 interaction with p300 and with type I HDACs and characterized the domains of Stat3 involved in these interactions. A series of domain-truncated Stat3 constructs were created and transfected with p300 or Hdac3 into 293T cells (fig. S2A). p300 interacts with Stat1 and Stat2 within both C-terminal TAD and N-terminal domain (*9*, *10*), as confirmed for Stat3 (Fig. 2B). The interaction of Stat3$^{1-585}$, which carries the linker domain, with p300 appears to be stronger than that of Stat3 domain mutants that lack the linker domain (Fig. 2B). We transfected Stat3 with individual HDAC factors in 293T cells and determined that Stat3 coimmunoprecipitated with Hdac1, Hdac2, and Hdac3 (Fig. 2C). Because Hdac3 displayed the strongest inhibitory effect on Stat3 deacetylase activity, we studied this interaction in

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more detail. Coimmunoprecipitation experiments revealed that Stat3 N-terminal region (amino acids 1 to 130) bears an Hdac3 docking site (Fig. 2D). $Stat3^{1-585}$ also showed strong interaction with Hdac3. Hdac1, Hdac2, and Hdac3 carry a homologous C-terminal region, which plays a regulatory role in HDAC catalytic activity (*16*). Stat3 immunoprecipitated with full-length or the C-terminal (amino acids 232 to 428) portion of Hdac3 but not with constructs of Hdac3 that lacked the C-terminal sequence (Fig. 2E). The interactions between endogenous Stat3 and type I HDAC factors in HeLa cells were detected by coimmunoprecipitation analysis (fig. S2B).

To identify the lysine residues of Stat3 acetylated by p300, we analyzed Stat3 truncation variants. Wild-type and truncated Stat3 constructs were transfected with p300 into 293T cells. Stat3 immunoprecipitates were prepared and analyzed with an antibody to acetylated lysine on Western blots. $Stat3^{1-722}$ lacking a C-terminal p300-docking site and wild-type Stat3 were comparably acetylated by p300. In contrast, neither $Stat3^{1-465}$ nor $Stat3^{1-585}$ was acetylated by p300 despite their interaction with p300 (Fig. 3A). Thus, the Stat3 C-terminal region from Met^{586} to Met^{770} appears to contain the lysine residues acetylated by p300. $Stat3^{465-770}$ contains 19 lysine residues, which are all conserved. In 293T cells, Stat3 acetylation by p300 was abrogated when a Lys^{685}-to-Arg substitution was introduced (Fig. 3B). Mutation of Lys^{685} to Arg in Stat3 did not cause any detectable changes in its complex formation either with p300 or with HDAC factors (*17*). In some transcription factors, lysine residues within the DNA binding domains are acetylated (*18*, *19*). We mutated each of these lysine residues identified within the Stat3 DNA binding domain and Stat3 N-terminal domain where Stat3 provides another binding site for p300. However, all such mutants were acetylated by p300 to levels comparable to those of wild-type Stat3, suggesting that it is unlikely that these lysine sites are acetylated by p300 (fig. S3A) (*17*).

Stat3 acetylation was further examined with an in vitro acetylation assay. Bacterially produced, purified, wild-type glutathione *S*-transferase (GST)–Stat3 but not GST-Stat3-$K^{685}R$ was acetylated by purified p300 or CBP (Fig. 3C). The antibody to acetylated lysine recognizes the acetylated peptide containing Stat3 Lys^{685} site and flanking sequences but not the unacetylated peptide

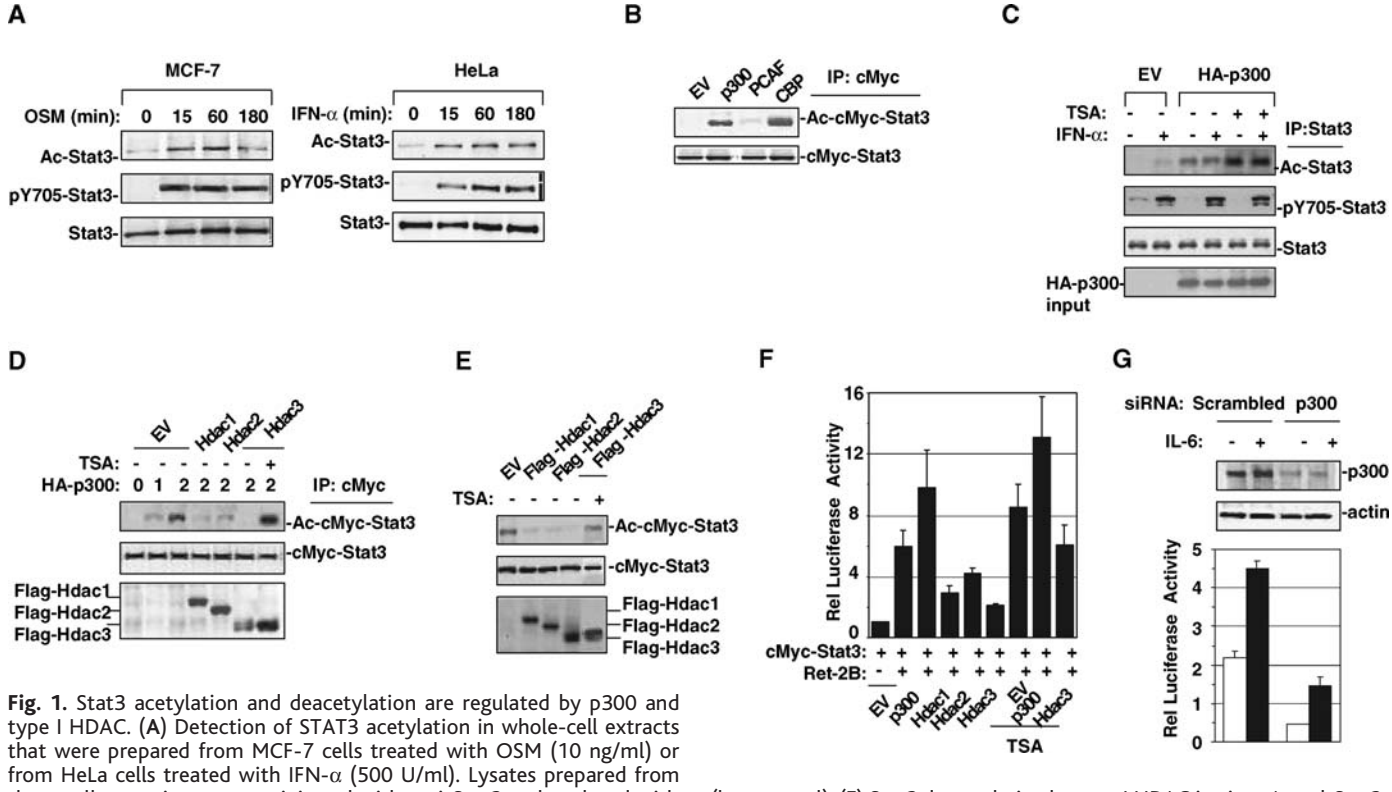

Fig. 1. Stat3 acetylation and deacetylation are regulated by p300 and type I HDAC. (**A**) Detection of STAT3 acetylation in whole-cell extracts that were prepared from MCF-7 cells treated with OSM (10 ng/ml) or from HeLa cells treated with IFN-α (500 U/ml). Lysates prepared from these cells were immunoprecipitated with anti-Stat3 and analyzed with antibodies to acetylated lysine (Cell Signaling Technology, Beverly, MA; catalog #9441), pY^{705}-Stat3, and Stat3. (**B**) Stat3 acetylation by transfection of Stat3 with p300, PCAF, or CBP. Lysates from 293T cells transfected with Myc-tagged Stat3 and p300, PCAF, or CBP were immunoprecipitated (IP) with anti-cMyc and analyzed with antibodies to acetylated lysine or Stat3. (**C**) TSA augments Stat3 acetylation. 293T cells transfected with or without HA-p300 were incubated with 0.2 μM TSA (Sigma, St. Louis, MO) for 2 hours followed by IFN-α treatment for an additional 30 min. Anti-Stat3 immunoprecipitates from the whole-cell extracts were analyzed with antibodies to acetylated lysine, pY^{705}-Stat3, Stat3, or HA. (**D**) Effect of type I HDAC on Stat3 acetylation in vivo. Whole-cell extracts were prepared from 293T cells that were transfected with HA-p300 at different doses (0, 1, 2 μg) or with 2 μg of HA-p300 DNA combined with 1 μg of vector encoding Hdac1, Hdac2, or Hdac3. Anti-cMyc immunoprecipitates were analyzed with antibodies to acetylated lysine or Stat3. In one condition, transfected 293T cells with p300 and Hdac3 were treated with 0.2 μM TSA for 2 hours. Expression levels of Hdac1, Hdac2, and Hdac3 were immunoblotted with anti-Flag (lower panel). (**E**) Stat3 deacetylation by type I HDAC in vitro. Acetyl-Stat3 proteins were immunoprecipitated with anti-cMyc from lysates of 293T cells transfected with p300 and cMyc-Stat3. Type I HDAC factors were immunoprecipitated with anti-Flag from 293T cells transfected with Flag-tagged Hdac1, Hdac2, or Hdac3. Stat3 deacetylation in vitro by type I HDAC was performed (*16*, *17*) and analyzed with antibodies to acetylated lysine, Stat3, or Flag. (**F**) Stat3 transcriptional activity was estimated in 293T cells transfected with p2xSIE-Luc, pRSV-β-gal, and RET-2B. In some samples, p300, Hdac1, Hdac2, and Hdac3 were included. Twenty-four hours after transfection, cells were treated with or without 0.2 μM TSA for 6 hours. The luciferase activity of each sample was normalized to β-galactosidase activity. Data are presented as means ± SD and represent results from three independent experiments. (**G**) p300 siRNA (AACCCCTCCTCTTCAGCACCA) or scramble siRNA was introduced into COS-1 cells for 24 hours, and SIE-luciferase reporter was transfected for an additional 24 hours followed by treatment with IL-6 for 6 hours. Whole extracts prepared from these cells were immunoblotted with antibodies to p300 or actin or analyzed for luciferase reporter activity. Data are presented as means ± SD and represent results from three independent experiments.

with the same sequence (fig. S3B). Additional evidence of Stat3 acetylation on Lys^{685} site was obtained by mass spectrometry analysis of acetylated Stat3 proteins purified from 293T cells transfected with Stat3 and p300 (*17*, *20*). Lys^{685} is highly conserved in Stat1, Stat3, Stat4, Stat5a, and Stat5b of different species. It resides in the highly hydrophilic region between the SH2 domain and TAD (fig. S3C). Secondary structural analysis predicts a small α helix immediately upstream of Lys^{685} residue and agrees with the crystal analysis of Stat1 (*3*).

To explore the role of Lys^{685} acetylation on Stat3 activity, we established cell lines by stably expressing either wild-type Stat3 or Stat3^{K685R} in PC3 cells, a human prostate cancer cell line lacking the *stat3* gene (*20*). Stat3^{K685R} was not acetylated, but both wild-type Stat3 and Stat3^{K685R} were tyrosine and serine phosphorylated and translocated into nuclei in response to OSM treatment (Fig. 4A and fig. S4). From analysis of the Stat1 and Stat3 crystal structures, we expected that the 35–amino acid segment between the SH2- and TAD-bearing Lys^{685} might be involved in Stat3 dimerization. Thus, we transfected cMyc-tagged and HA-tagged Stat3 into PC3 cells. Anti-cMyc immunoprecipitates from the whole-cell extracts prepared from these

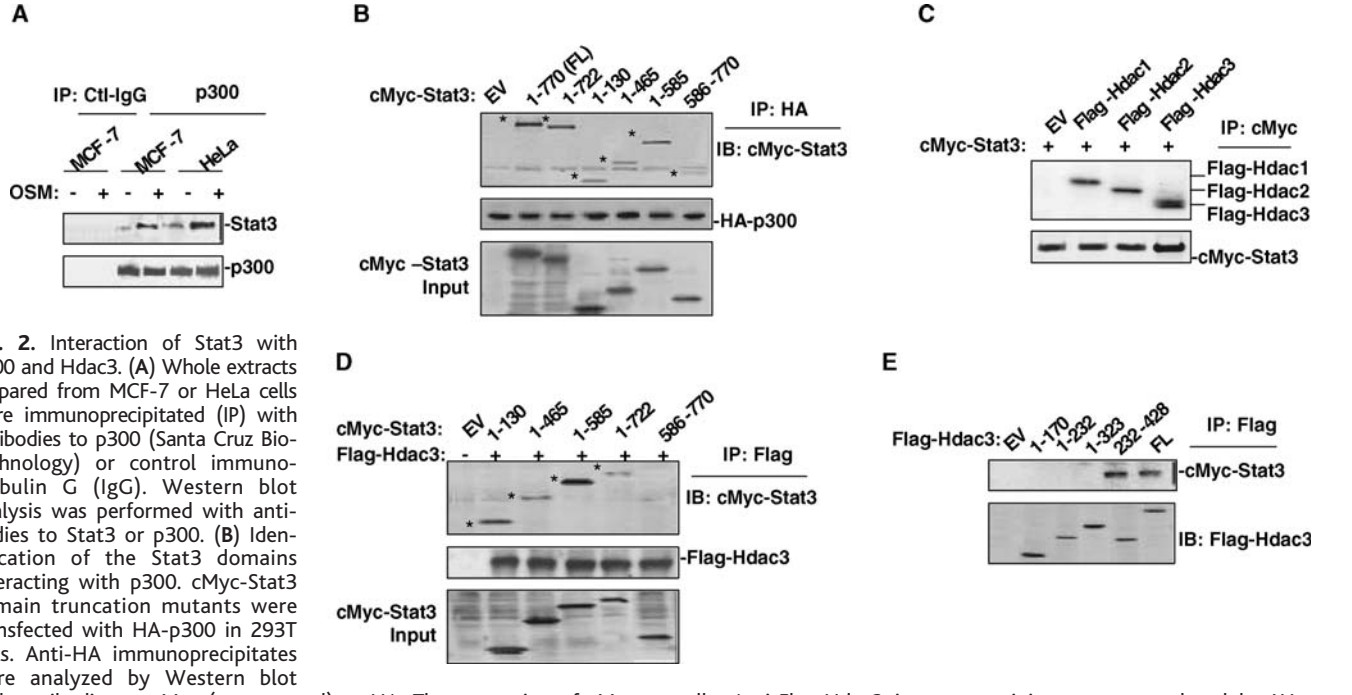

Fig. 2. Interaction of Stat3 with p300 and Hdac3. (**A**) Whole extracts prepared from MCF-7 or HeLa cells were immunoprecipitated (IP) with antibodies to p300 (Santa Cruz Biotechnology) or control immunoglobulin G (IgG). Western blot analysis was performed with antibodies to Stat3 or p300. (**B**) Identification of the Stat3 domains interacting with p300. cMyc-Stat3 domain truncation mutants were transfected with HA-p300 in 293T cells. Anti-HA immunoprecipitates were analyzed by Western blot with antibodies to cMyc (upper panel) or HA. The expression of cMyc-Stat3 is shown in the bottom panel. (**C**) Stat3 associates with Hdac1, Hdac2, and Hdac3 in 293T cells. Anti-cMyc immunoprecipitates were subjected to Western blot analysis with antibodies to Flag or cMyc. (**D**) Identification of the Stat3 domains interacting with Hdac3. cMyc-Stat3 domain truncation mutants were transfected with Flag-Hdac3 in 293T cells. Anti-Flag-Hdac3 immunoprecipitates were analyzed by Western blot with anti-cMyc. (**E**) Identification of the Hdac3 domains interacting with Stat3. A series of Flag-Hdac3 with truncation mutations were constructed and transfected with cMyc-Stat3 into 293T cells. Anti-Flag immunoprecipitates were analyzed in Western blot with antibodies to cMyc or Flag.

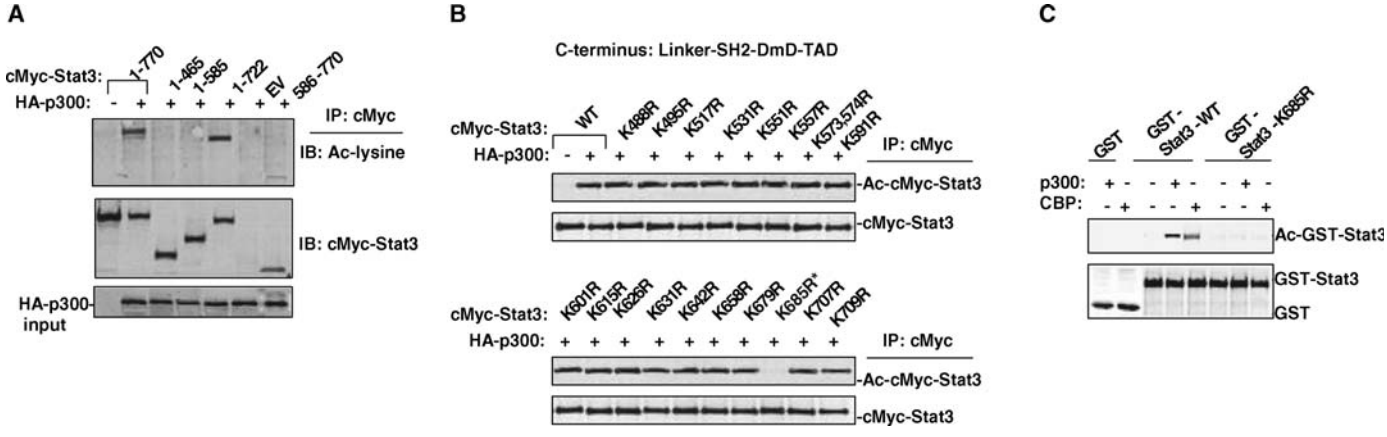

Fig. 3. Stat3 is acetylated on Lys^{685} site by p300. (**A**) Deletion analysis of Stat3 acetylation. A series of cMyc-tagged Stat3 with deletion mutations were cotransfected with HA-p300 in 293T cells. Anti-Stat3 immunoprecipitates were subjected to immunoblotting (IB) analysis with antibodies to acetyl-lysine or cMyc. HA-p300 levels are shown in the bottom panel. (**B**) p300-mediated acetylation of Stat3 with a K→R substitution. All Stat3 constructs with a K→R substitution were prepared by site-directed mutagenesis and transfected with p300 in 293T cells. cMyc-tagged Stat3 proteins were immunoprecipitated with anti-cMyc and analyzed with antibodies to acetyl lysine or cMyc. (**C**) K^{685}→R substitution abolishes STAT3 acetylation by p300 or CBP in vitro. Bacterial purified GST, wild-type GST-Stat3, or GST-Stat3-K^{685}R mutant proteins were incubated with purified p300 HAT protein domain or CBP protein in acetylation reaction buffer (*17*), followed by Western blot analysis with antibodies to acetylated lysine.

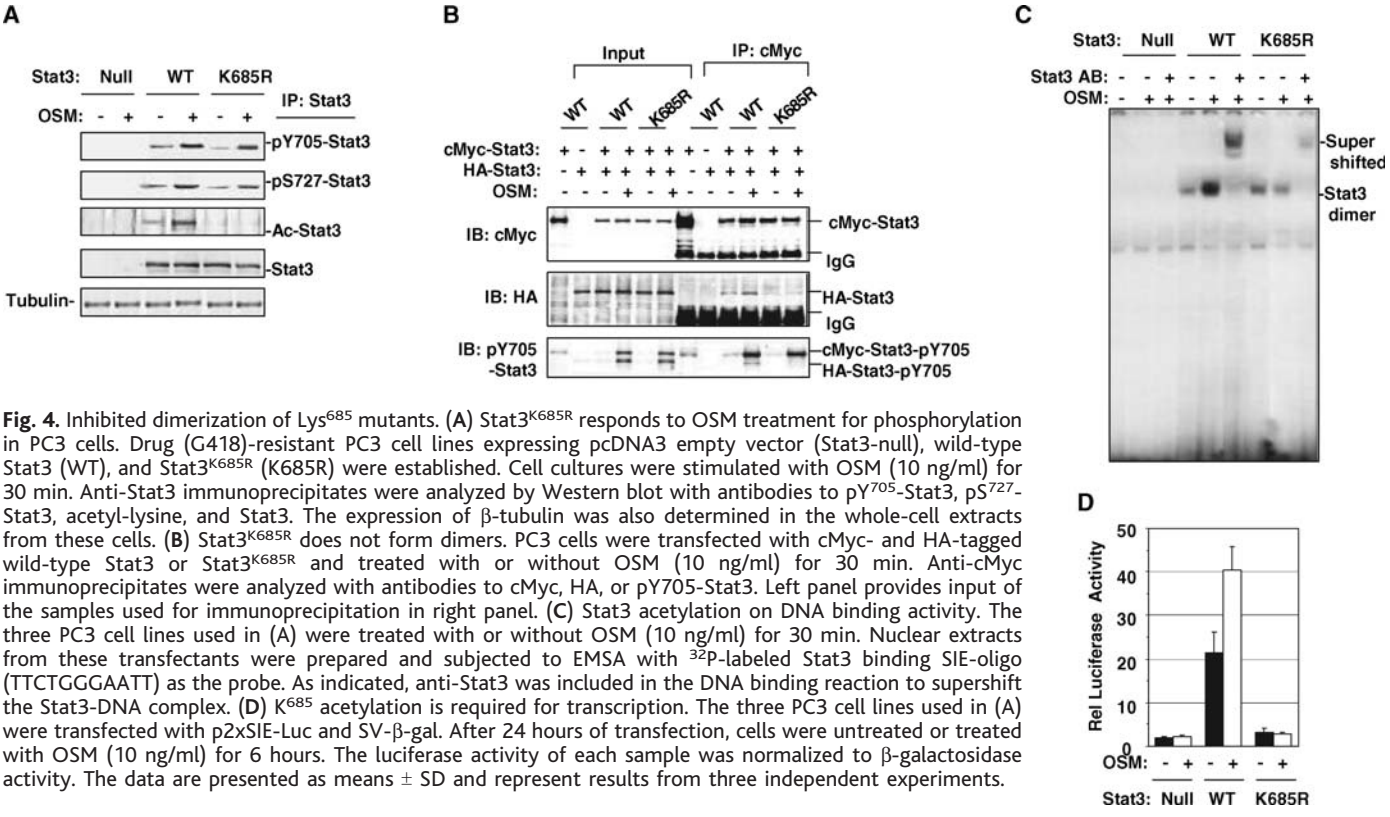

Fig. 4. Inhibited dimerization of Lys^{685} mutants. (**A**) Stat3^{K685R} responds to OSM treatment for phosphorylation in PC3 cells. Drug (G418)-resistant PC3 cell lines expressing pcDNA3 empty vector (Stat3-null), wild-type Stat3 (WT), and Stat3^{K685R} (K685R) were established. Cell cultures were stimulated with OSM (10 ng/ml) for 30 min. Anti-Stat3 immunoprecipitates were analyzed by Western blot with antibodies to pY^{705}-Stat3, pS^{727}-Stat3, acetyl-lysine, and Stat3. The expression of β-tubulin was also determined in the whole-cell extracts from these cells. (**B**) Stat3^{K685R} does not form dimers. PC3 cells were transfected with cMyc- and HA-tagged wild-type Stat3 or Stat3^{K685R} and treated with or without OSM (10 ng/ml) for 30 min. Anti-cMyc immunoprecipitates were analyzed with antibodies to cMyc, HA, or pY705-Stat3. Left panel provides input of the samples used for immunoprecipitation in right panel. (**C**) Stat3 acetylation on DNA binding activity. The three PC3 cell lines used in (A) were treated with or without OSM (10 ng/ml) for 30 min. Nuclear extracts from these transfectants were prepared and subjected to EMSA with ^{32}P-labeled Stat3 binding SIE-oligo (TTCTGGGAATT) as the probe. As indicated, anti-Stat3 was included in the DNA binding reaction to supershift the Stat3-DNA complex. (**D**) K^{685} acetylation is required for transcription. The three PC3 cell lines used in (A) were transfected with p2xSIE-Luc and SV-β-gal. After 24 hours of transfection, cells were untreated or treated with OSM (10 ng/ml) for 6 hours. The luciferase activity of each sample was normalized to β-galactosidase activity. The data are presented as means ± SD and represent results from three independent experiments.

PC3 transfectants were subjected to Western blot analysis with anti-HA or anti–tyrosine-phosphorylated Stat3 (Fig. 4B). In PC3 cells transfected with cMyc-tagged and HA-tagged wild-type Stat3, coimmunoprecipitation detected the association, which was stabilized by OSM treatment, between these two forms of Stat3 (Fig. 4B). However, when HA-Stat3^{K685R} and cMyc-Stat3^{K685R} were tested, the association between these two forms of Stat3 was undetectable, even though they were tyrosine phosphorylated upon OSM treatment (Fig. 4B). Thus, acetylation of Lys^{685} appears to be critical for Stat3 to form dimers. Because dimerized STAT proteins bind to DNA, we evaluated Stat3-DNA complex formation in these two cell lines using ^{32}P-labeled sis-inducible element (SIE) as the probe in electrophoretic mobility shift assay (EMSA). OSM treatment markedly induced formation of Stat3-DNA complex in PC3 cells expressing wild-type Stat3, whereas in PC3 cells expressing Stat3^{K685R}, we detected a basal level of complex formation, which was not increased in cells treated with OSM (Fig. 4C). Stat3-dependent luciferase activity assay revealed that introduction of wild-type Stat3 but not of Stat3^{K685R} restored the response of PC3 cells to OSM (Fig. 4D).

The effect of Stat3 acetylation on cell growth in vivo was further examined in PC3 cells transfected with empty vector, wild-type Stat3, or Stat3^{K685R}. Stat3 activation regulates genes involved in cell growth and cell survival, including *cyclin D1*, *bcl-X$_L$*, and c-*myc* (*21*, *22*). In parental PC3 cells or PC3 cells expressing empty vector, cyclin D1, cMyc, and Bcl-X$_L$ proteins were nearly undetectable in the presence or absence of OSM treatment (Fig. 5A). In PC3 cells expressing wild-type Stat3, OSM treatment stimulated the expression of these three proteins, as determined by Western blot analysis (Fig. 5A). In contrast, in PC3 cells expressing stable Stat3^{K685R}, OSM treatment did not alter the expression of these three proteins (Fig. 5A). TSA, the HDAC inhibitor, alone induced expression of cyclin D1, but only in PC3 cells expressing wild-type Stat3 (fig. S5). We also analyzed cell growth in these three types of PC3 cells. [^{3}H]thymidine incorporation assays revealed a dose-dependent stimulation of growth by OSM in wild-type Stat3-expressing PC3 cells, whereas parental cells or cells expressing Stat3^{K685R} showed little apparent effect of OSM on DNA replication (Fig. 5B). Consistent expression of wild-type Stat3 but not of Stat3-K^{685}R accelerated cell cycle progression in response to OSM treatment in PC3 cells after synchronization with aphidicolin (Fig. 5C). Inconsistent effects of Stat3 on cell growth in different cell types have been reported (*21*–*23*), but our results demonstrate that expression of Stat3 is required for transcriptional activation of genes involved in cell cycle progression and for stimulating cell growth after exposure to OSM in PC3 cells.

GKX$_2$P (where G is glycine, P is proline, and X represents any amino acid), or GK, is a preferred sequence in histone or other protein substrates for acetylation by HAT (*19*, *24*). In STAT, the acetylated lysine is relatively conserved as a G(S)KX$_{3-5}$P sequence. Recently, genes carrying STAT-type linker-SH2 domains within their C-terminal regions have been discovered in *Arabidopsis*, indicating that the linker-SH2 domain of STATs was fully developed before the divergence of plant and animal kingdoms (*25*). In animals, the linker-SH2 domain of STAT extends into DmD, which harbors the GKX$_{3-5}$P motif and the phosphotyrosine motif GYXK followed by TAD, which harbors the phosphoserine motif PMSP. Stat2 and Stat6 represent the most divergent STAT members in evolution (*26*), and both lack GKX$_{3-5}$P and PMSP motifs in the C-terminal region. It is possible that another lysine residue within this domain is acetylated in these two STATs. Although in animals STAT evolved to have DmD-TAD, both GKX$_{3-5}$P and PMSP motifs might have evolved after the appearance of the GYXK motif. STAT acetylation may well be analogous to STAT tyrosine phosphorylation. However, neither Tyr^{705} phosphorylation alone nor Lys^{685} acetylation alone seems to be sufficient for Stat3 activation. Acetylation of Lys^{685} may change the local charge of DmD, form a platform for the interaction, and strengthen the dimer formation (fig. S6). Crystal analysis revealed that STAT (Stat1 and Stat3), nuclear factor NF-κB (Rel A), and p53 bind DNA in a similar topology (*2*, *3*). In p53, C-terminal acetylation regulates p53 DNA binding activity, presumably via a

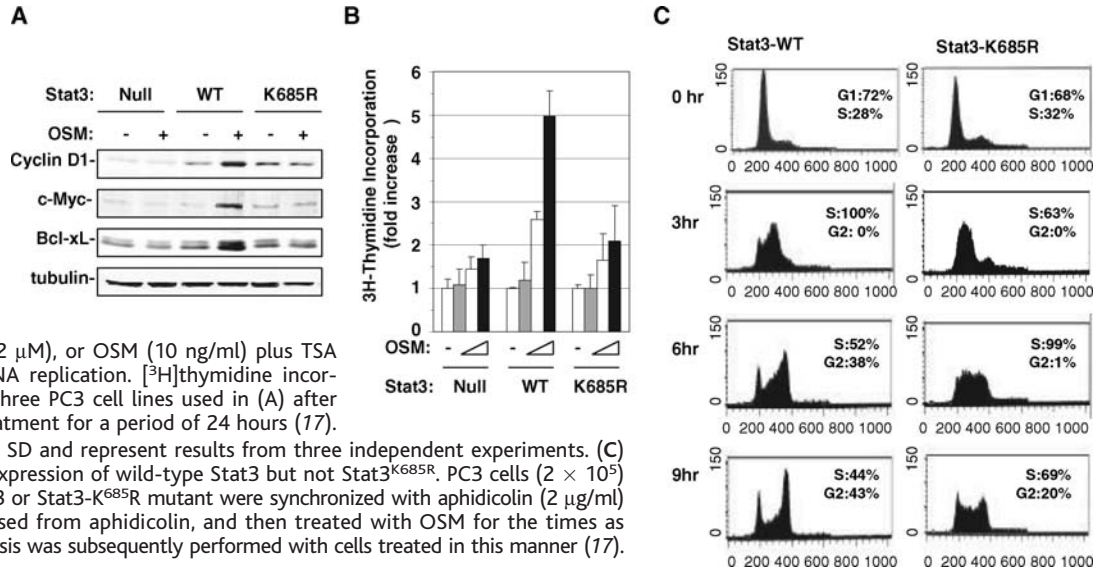

Fig. 5. Lys[685] acetylation in Stat3 stimulates cell proliferation. (**A**) Lys[685] acetylation is critical for cell cycle–related gene expression. Western blot analysis was performed with antibodies to cyclin D1 (Santa Cruz Biotechnology), cMyc (Santa Cruz Biotechnology), Bcl-$X_{S/L}$ (Santa Cruz Biotechnology), and β-tubulin in PC3 cells expressing empty vector (null), wild-type Stat3 (WT), or Stat3[K685R] (K685R) treated with OSM (10 ng/ml), TSA (0.2 μM), or OSM (10 ng/ml) plus TSA (0.2 μM) for 12 hours. (**B**) DNA replication. [^{3}H]thymidine incorporation was analyzed in the three PC3 cell lines used in (A) after OSM (5, 10, and 20 ng/ml) treatment for a period of 24 hours (*17*). Data are presented as means ± SD and represent results from three independent experiments. (**C**) Accelerated S-phase entry by expression of wild-type Stat3 but not Stat3[K685R]. PC3 cells (2×10^5) expressing either wild-type Stat3 or Stat3-K[685]R mutant were synchronized with aphidicolin (2 μg/ml) for a period of 12 hours, released from aphidicolin, and then treated with OSM for the times as indicated. Flow cytometric analysis was subsequently performed with cells treated in this manner (*17*).

conformational change of p53 (*18*). In Rel A, acetylation of lysine sites in the dimerization domain augments Rel A's DNA binding activity (*27*). Our results raise the possibility that acetylation of NF-κB and p53 promotes transcriptional activity by a mechanism similar to the acetylation of Stat3. In HeLa cells, all three type I HDACs are associated with Stat3, either in the cytoplasm or nucleus. As such, unlike NF-κB, which responds only to Hdac-3 for deacetylation (*28*), type I HDACs function as Stat3-deacetylase factors that, when associated with Stat3, either terminate Stat3's stimulatory effect on transcription or maintain Stat3 in a deacetylated form. Given the role of CBP/p300 as a coactivator for different STAT members, it is possible that all STAT family members are tightly regulated by the acetylation and deacetylation cycle.

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Figs. S1 to S6

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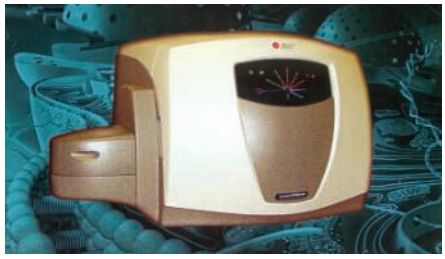

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Author Index Volume 306
October–December 2004

A and B

AAAS News & Notes: AAAS members elected as fellows. p825 29 Oct 2004. Basking in the light of superstars. p824 29 Oct 2004. Global praise for AAAS health rights manual. p825 29 Oct 2004. A gritty crash course in the ways of Washington. p2204 24 Dec 2004. Herman and Victor Wouk: a brotherhood of discovery. p1496 26 Nov 2004. Omenn sees "grand challenges and great opportunities." p2204 24 Dec 2004. Results of the 2004 election of AAAS officers. p2205 24 Dec 2004. "Serious concerns" with electronic voting. p1497 26 Nov 2004. Spreading the word: "Science. It's everywhere." p1497 26 Nov 2004.

Abergel, Chantal. *See* Raoult, Didier.

Abmayr, Susan M. *See* Kusch, Thomas.

Abraham, Spencer: The Bush Administration and climate change (letter). p2041 17 Dec 2004.

Achermann, C. *See* Readhead, A. C. S.

Acosta, Juana Luz Solis. *See* D'Hondt, Steven.

Acuña, C. *See* Thomas, R.

Adai, Alex T. *See* Lee, Insuk.

Adams, William M.; Aveling, Ros; Brockington, Dan; Dickson, Barney; Elliott, Jo; Hutton, Jon; Roe, Dilys; Vira, Bhaskar; Wolmer, William: Biodiversity conservation and the eradication of poverty. p1146 12 Nov 2004.

Addadi, Lia. *See* Politi, Yael.

Adelman, Carol C.: Support for Steiger's policies (letter). p977 5 Nov 2004.

Adkins, Jess F. and Pasquero, Claudia: Deep ocean overturning—then and now (perspective). p1143 12 Nov 2004.

Adler, Elizabeth M. *See* Ray, L. Bryan.

Adolphs, Ralph and Woodward, James: review of *The Physiology of Truth*, by Jean-Pierre Changeux. p1684 3 Dec 2004.

Agosta, Salvatore J. and Dunham, Arthur E.: Comment on "How the horned lizard got its horns" (technical comment). p230 8 Oct 2004. www.sciencemag.org/cgi/content/full/306/5694/230a.

Ahmed, Serge H.: Addiction as compulsive reward prediction (perspective). p1901 10 Dec 2004.

Aiello, Ivano W. *See* D'Hondt, Steven.

Ainslie, George and Monterosso, John: A marketplace in the brain? (perspective). p421 15 Oct 2004.

Akhmedov, Alexander. *See* Ricci, Romeo.

Akins, T. *See* Thomas, R.

Alba, David M. *See* Moyà-Solà, Salvador.

Alberta, John A. *See* Arnett, Heather A. *See also* Gray, Paul A.

Alivisatos, A. Paul. *See* Son, Dong Hee.

Allakhverdov, Andrey and Pokrovsky, Vladimir (news): Putin: reform begins at home. p957 5 Nov 2004. Russia, reluctantly, backs Kyoto. p209 8 Oct 2004.

Allard, John. *See* Liao, Guochun.

Allen, Andrew E. *See* Armbrust, E. Virginia.

Allen, Rebecca L.; Bittner-Eddy, Peter D.; Grenville-Briggs, Laura J.; Meitz, Julia C.; Rehmany, Anne P.; Rose, Laura E.; Beynon, Jim L.: Host-parasite coevolutionary conflict between *Arabidopsis* and downy mildew. p1957 10 Dec 2004.

Alley, Richard B. *See* Schrag, Daniel P.

Allis, C. David. *See* Wang, Yanming.

Almers, Wolfhard. *See* An, Seong J.

Alonso, Jose M. and Stepanova, Anna N.: The ethylene signaling pathway. p1513 26 Nov 2004.

Altamirano, P. *See* Readhead, A. C. S.

Alter, Blanche P. *See* Lan, Qing.

Altschuler, Steven J. *See* Perlman, Zachary E.

Amano, Atsuo. *See* Nakagawa, Ichiro.

An, Seong J. and Almers, Wolfhard: Tracking SNARE complex formation in live endocrine cells. p1042 5 Nov 2004.

Anderson, R. C. *See* Arvidson, R. E. *See also* Rieder, R. *See also* Soderblom, L. A.

Anderson, Tim. *See* Roper, Cally.

Anderson, Travis M.; Neiwert, Wade A.; Kirk, Martin L.; Piccoli, Paula M. B.; Schultz, Arthur J.; Koetzle, Thomas F.; Musaev, Djamaladdin G.; Morokuma, Keiji; Cao, Rui; Hill, Craig L.: A late-transition metal oxo complex: $K_7Na_9[O{=}Pt^{IV}(H_2O)L_2]$, L = $[PW_9O_{34}]^{9-}$. p2074 17 Dec 2004.

Ando, Ryoko; Mizuno, Hideaki; Miyawaki, Atsushi: Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. p1370 19 Nov 2004.

Andreadis, T. G. *See* Spielman, A.

Andrew, P. and Barnes, W. L.: Energy transfer across a metal film mediated by surface plasmon polaritons. p1002 5 Nov 2004.

Andronova, Natasha. *See* Yohe, Gary.

Ané, Cécile. *See* Driskell, Amy C.

Ansorge, Mark S.; Zhou, Mingming; Lira, Alena; Hen, René; Gingrich, Jay A.: Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. p879 29 Oct 2004.

Antoniou, Antonis. *See* Easton, Douglas F.

Anwar, S. *See* Christensen, P. R.

Apel, Klaus. *See* Wagner, Daniela.

Apicella, Michael A. *See* Koropatnick, Tanya A.

Apperson, C. S. *See* Spielman, A.

Apt, Kirk E. *See* Armbrust, E. Virginia.

Arad, Talmon. *See* Politi, Yael.

Arcese, Peter. *See* Brashares, Justin S.

Archibald, John M. *See* Keeling, Patrick J.

Arendt, Detlev; Tessmar-Raible, Kristin; Snyman, Heidi; Dorresteijn, Adriaan W.; Wittbrodt, Joachim: Ciliary photoreceptors with a vertebrate-type opsin in an invertebrate brain. p869 29 Oct 2004.

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Arneson, H. M. *See* Bell, J. F., III.

Arnett, Heather A.; Fancy, Stephen P. J.; Alberta, John A.; Zhao, Chao; Plant, Sheila R.; Kaing, Sovann; Raine, Cedric S.; Rowitch, David H.; Franklin, Robin J. M.; Stiles, Charles D.: bHLH transcription factor Olig1 is required to repair demyelinated lesions in the CNS. p2111 17 Dec 2004.

Arnsten, A. F. T. *See* Birnbaum, S. G.

Aron, Serge. *See* Pearcy, Morgan.

Arvidson, R. *See* Herkenhoff, K. E.

Arvidson, R. E. *See* Bell, J. F., III. *See also* Christensen, P. R. *See also* Klingelhöfer, G. *See also* Soderblom, L. A. *See also* Squyres, S. W.

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Attardi, Giuseppe. *See* Fish, Jennifer.

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Blauvelt, Andrew. *See* Lederman, Michael M.
Bloem, J. *See* Dolfing, J.
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Bussemaker, Harmen J. *See* Stolc, Viktor.
Bustamante, Carlos D. *See* Shimizu, Kentaro K.
Bustos, R. *See* Readhead, A. C. S.

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Cabrol, N. A. *See* Soderblom, L. A. *See also* Squyres, S. W.
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Calvin, W. M. *See* Christensen, P. R.
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Canals, Miquel. *See* Martrat, Belen.
Cantu, Carlos, III. *See* Zhou, Dapeng.
Cao, Qiong. *See* Özcan, Umut.
Cao, Rui. *See* Anderson, Travis M.
Cao, Yuzhu. *See* Xia, Qingyou.
Cappa, Christopher D. *See* Smith, Jared D.
Carbone, Chris. *See* Jetz, Walter.
Cardinale, Bradley J. *See* Solan, Martin.
Carell, Thomas. *See* Mees, Alexandra.
Carlson, Jane. *See* Houghton, JeanMarie.
Carlstrom, J. E. *See* Readhead, A. C. S.
Caron, Marc G. *See* Wilbanks, Alyson M.
Carr, M. H. *See* Squyres, S. W.
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Cartwright, J. K. *See* Readhead, A. C. S.
Casanovas-Vilar, Isaac. *See* Moyà-Solà, Salvador.
Casassa, G. *See* Thomas, R.
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Celotta, Robert J. *See* Stroscio, Joseph A.
Chaal, Balbir K. *See* Armbrust, E. Virginia.
Chai, Chunli. *See* Xia, Qingyou.
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Chang, Amy. *See* Handelsman, Jo.
Chanock, Stephen. *See* Lan, Qing.
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Cheng, Le-ping. *See* Gray, Paul A.
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Chuang, Chia-Chin. *See* Sfeir, Matthew Y.
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Clark, B. C. *See* Rieder, R. *See also* Soderblom, L. A. *See also* Squyres, S. W.
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Claverie, Jean-Michel. *See* Raoult, Didier.
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Gentile, Jim. *See* Handelsman, Jo.
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Gerdes, Kenn. *See* Møller-Jensen, Jakob.
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Ghosal, D. *See* Daly, M. J.
Ghosh, A. *See* Lemmon, M. T.
Ghosh, Amitabha. *See* Smith, Michael D.
Ghosh, Manik C. *See* Meyron-Holtz, Esther G.
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Gibbs, Richard A. *See* Nelson, David L.
Gibney, C. A. *See* Nelson, D. E.
Gilbert, M. Thomas P. *See* Shapiro, Beth.
Gill, P. *See* Margolis, H. S.
Gill, Peter and Hagelberg, Erika: Ongoing controversy over Romanov remains (letter). p407 15 Oct 2004.
Gilles, Mary K. *See* Maria, Steven F.
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Gilmore, Michael S. *See* Coburn, Phillip S.
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Gingrich, Jay A. *See* Ansorge, Mark S.
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Glotzer, Sharon C.: Some assembly required (perspective). p419 15 Oct 2004.
Gnau, Petra. *See* Mees, Alexandra.
Gobeske, K. T. *See* Birnbaum, S. G.
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Goklany, Indur M.: Climate change and malaria (letter). p55 1 Oct 2004.
Goldberg, Gregory. *See* Saffarian, Saveez.
Goldenring, James R. *See* Houghton, JeanMarie.
Goldman, William E. *See* Koropatnick, Tanya A.
Gologan, Bogdan. *See* Takáts, Zoltán.
Golombek, M. *See* Bell, J. F., III. *See also* Squyres, S. W.
Golombek, M. P. *See* Arvidson, R. E. *See also* Soderblom, L. A.
Gonzalez, Tania N. *See* Wickens, Marvin.
González-Rouco, Fidel. *See* von Storch, Hans.
Good, Robert T. *See* Marti, Matthias.
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Goodman, Howard M. *See* Shen, Binzhang.
Goodman, Ian P. *See* Pighin, Jamie A.
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Gorevan, S. *See* Arvidson, R. E. *See also* Squyres, S. W.
Görgün, Cem. *See* Özcan, Umut.
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Gower, David J. *See* Bossuyt, Franky.
Gowland, Ian. *See* Wadsworth, Jonathan D. F.
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Green, A. G. *See* Grigera, S. A.
Green, Beverley R. *See* Armbrust, E. Virginia.
Green, Lance D. *See* Knight, Alec.
Greenberg, Michael E. *See* Gray, Paul A.

Greenberg, Richard. *See* Richardson, James E.
Greene, Mark H. *See* Wacholder, Sholom.
Greengard, P. *See* Rakhilin, S. V.
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Gritsai, Y. *See* Bargheer, M.
Gropp, Robert E.: Budget cuts affecting natural history (letter). p811 29 Oct 2004.
Grosch, C. E. *See* Gargett, A.
Grose, Jacob E. *See* Pasupathy, Abhay N.
Grotzinger, J. *See* Bell, J. F., III. *See also* Herkenhoff, K. E. *See also* Squyres, S. W.
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Guinness, E. *See* Bell, J. F., III.
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Gütlich, P. *See* Klingelhöfer, G.

H and I

Haas, Wolfgang. *See* Coburn, Phillip S.
Habegger, L. J. *See* Whicker, F. W.
Hadi, Masood Z. *See* Armbrust, E. Virginia.
Haegel, Nancy. *See* Deardorff, Don.
Haemmerle, Guenter. *See* Zimmermann, Robert.
Hagelberg, Erika. *See* Gill, Peter.
Haggblom, Candy I. *See* Crabbe, Laure.
Hahn, Thomas T. G. *See* Petersen, Carl C. H.
Haile, Robert W. *See* Easton, Douglas F.
Hajdas, Irka. *See* Broecker, Wallace.
Halasz, Gabor. *See* Stolc, Viktor.
Haldar, Kasturi. *See* Hiller, N. Luisa.
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Haley, Benjamin. *See* Tomari, Yukihide.
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Hall, S. H. *See* O'Rand, M. G.
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Hamann, Thorsten. *See* Somerville, Chris.
Hamilton, V. E. *See* Christensen, P. R.
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Hanusse, P. *See* Loudet, J. C.
Hardikar, Anandwardhan A. *See* Gershengorn, Marvin C.
Harding, L. B. *See* Townsend, D.
Harington, C. Richard. *See* Shapiro, Beth.
Hariri, Ali. *See* Wilson, Frederick H.
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Harrington, L. C. *See* Spielman, A.
Harris, Lasana T. *See* Fiske, Susan T.
Harrison, T. M. *See* Becker, L.
Harrison, T. Mark. *See* Turner, Grenville.
Harrison, Travis. *See* Hiller, N. Luisa.
Hartge, Patricia. *See* Wacholder, Sholom.
Hartl, Daniel L. *See* Kulathinal, Rob J.
Hartley, Oliver. *See* Lederman, Michael M.
Haskin, L. *See* Squyres, S. W.
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Hayes, A. G. *See* Bell, J. F., III. *See also* Christensen, P. R. *See also* Herkenhoff, K. E.
Hayes, Richard B. *See* Lan, Qing.
He, Dandan. *See* Xia, Qingyou.
He, Shigang. *See* Zhang, Ya-Ping.
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Hellsten, Uffe. *See* Armbrust, E. Virginia.
Hempstead, Barbara L. *See* Pang, Petti T.
Hen, René. *See* Ansorge, Mark S.
Henderson, D. A. *See* Maier, R. M.
Heneman, Burr. *See* Pikitch, Ellen K.
Hennecke, Ulrich. *See* Mees, Alexandra.
Herbert, Gregory S. *See* Dietl, Gregory P.
Hering, J. G. *See* Malasarn, D.
Herken, Gregg: review of *J. Robert Oppenheimer and the American Century*, by David C. Cassidy. p1137 12 Nov 2004.
Herkenhoff, K. E. *See* Arvidson, R. E. *See also* Bell, J. F., III. *See also* Soderblom, L. A. *See also* Squyres, S. W.
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Hermetter, Albin. *See* Zimmermann, Robert.
Hernández, José V.; Kay, Euan R.; Leigh, David A.: A reversible synthetic rotary molecular motor. p1532 26 Nov 2004.
Herreman, Tine. *See* Stolc, Viktor.
Hersberger, Martin. *See* Ricci, Romeo.
Hertel, Ingolf V. *See* Schultz, Thomas.
Hertenberger, R. *See* Reichart, P.
Hess, M. *See* Daly, M. J.
Hester, Keith C. *See* Florusse, Louw J.
Hideg, Eva. *See* Wagner, Daniela.
Higashi, Toshihiro. *See* Imanishi, Yuichi.
Higgins, Paul A. T.: The Bush Administration and climate change (letter). p2041 17 Dec 2004.
Hildebrand, Mark. *See* Armbrust, E. Virginia.
Hill, Andrew F. *See* Wadsworth, Jonathan D. F.
Hill, Christopher. *See* Baumeister, Wolfgang.
Hill, Craig L. *See* Anderson, Travis M.
Hill, Geoffrey E.: A head start for some redstarts (perspective). p2201 24 Dec 2004.
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Hinrichs, Kai-Uwe. *See* D'Hondt, Steven.
Hinton, T. G. *See* Whicker, F. W.
Hochstrasser, Mark. *See* Baumeister, Wolfgang.
Hodell, David A. *See* Martrat, Belen.
Hoffmann, Dieter H. H. *See* Zioutas, Konstantin.
Hofreiter, Michael. *See* Matheus, Paul.
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Hogle, James M. *See* Guogas, Laura M.
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Holden, Constance. *See* Bhattacharjee, Yudhijit.
Holland, Greg. *See* Turner, Grenville.

Holm, Nils G. *See* D'Hondt, Steven.
Holzapfel, W. L. *See* Readhead, A. C. S.
Hone, J. *See* Sfeir, Matthew Y.
Honjo, T. *See* Begum, N. A.
Hood, Leroy; Heath, James R.; Phelps, Michael E.; Lin, Biaoyang: Systems biology and new technologies enable predictive and preventative medicine. p640 22 Oct 2004.
Hooykaas, Paul J. J. *See* Friml, Jiří.
Hopper, John L. *See* Easton, Douglas F.
Horton, Benjamin P. *See* Sawai, Yuki.
Horton, C. A. *See* Nelson, D. E.
Hoshi, Toshinori and Lahiri, Sukhamay: Oxygen sensing: it's a gas! (perspective). p2050 17 Dec 2004.
Hotamisligil, Gökhan S. *See* Özcan, Umut.
Hou, Yong. *See* Xia, Qingyou.
Houde, Ed. *See* Pikitch, Ellen K.
Houghton, JeanMarie; Stoicov, Calin; Nomura, Sachiyo; Rogers, Arlin B.; Carlson, Jane; Li, Hanchen; Cai, Xun; Fox, James G.; Goldenring, James R.; Wang, Timothy C.: Gastric cancer originating from bone marrow–derived cells. p1568 26 Nov 2004.
House, Christopher H. *See* D'Hondt, Steven.
Hua, Sujun. *See* Stolc, Viktor.
Huang, G. *See* Margolis, H. S.
Huang, Haiyan. *See* Xia, Qingyou.
Huang, Limin. *See* Sfeir, Matthew Y.
Huang, Xiangang. *See* Xia, Qingyou.
Huang, Yanqing. *See* Xia, Qingyou.
Hubbard, M. Y. H. *See* Bell, J. F., III.
Huber, Robert. *See* Baumeister, Wolfgang.
Hudson, Thomas J. *See* Pastinen, Tomi.
Hudspeth, Kelly. *See* Zhou, Dapeng.
Hughes, Steven M. *See* Son, Dong Hee.
Huguet, Elisabeth. *See* Espagne, Eric.
Hulvey, Kristin B. *See* Zavaleta, Erika S.
Humayun, Munir; Qin, Liping; Norman, Marc D.: Geochemical evidence for excess iron in the mantle beneath Hawaii. p91 1 Oct 2004.
Hunten, D. M. *See* Sprague, A. L.
Hunter, Tony. *See* York, John D.
Hushka, Leslie J. *See* Silbergeld, Ellen.
Hutton, Jon. *See* Adams, William M.
Hviid, S. *See* Squyres, S. W.
Hviid, S. F. *See* Herkenhoff, K. E.
Iasky, R. *See* Becker, L.
Idrobo, J. C. *See* Ziegler, A.
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Ignatiev, Nikolai. *See* Formisano, Vittorio.
Ihekwaba, A. E. C. *See* Nelson, D. E.
Iijima, Sumio. *See* Hata, Kenji.
Iles, David E. *See* Williams, Sandile E. J.
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Inagaki, Fumio. *See* D'Hondt, Steven.
Inamura, Yasuhiro. *See* Katayama, Yoshinori.
Innan, Hideki. *See* Gao, Li-zhi.
Insel, Thomas R. *See* Austin, Christopher P.
Irion, Robert (news): Astronomers eager for a swift new vision of the universe. p214 8 Oct 2004. Gamma ray bursts: new cosmic rulers? p215 8 Oct 2004.
Ittekkot, Venugopalan. *See* Jennerjahn, Tim C.
Ivanov, Boris A. *See* Renne, Paul R.
Iverson, Brent L. *See* Deardorff, Don.
Iwakoshi, Neal N. *See* Özcan, Umut.
Izard, Véronique. *See* Pica, Pierre.

J and K

Jackson, Meyer B. *See* Han, Xue.
Jackson, Peter. *See* Baumeister, Wolfgang.
Jaeger, Luc. *See* Chworos, Arkadiusz.
Jaffe, Laurinda A. *See* Mehlmann, Lisa M.
Jagannadha Rao, A. *See* O'Rand, M. G.
Jäger, Martina. *See* Leinders-Zufall, Trese.
Janes, D. M. *See* Sprague, A. L.
Janney, Philip E. *See* Dauphas, Nicolas.
Jansen, Vincent A. A. *See* Bonsall, Michael B.
Janzen, Daniel H. *See* Bawa, Kamaljit S.
Jasny, Barbara R. and Roberts, Leslie (news): Solving gene expression. p629 22 Oct 2004.
Jefferts, S. R. *See* Diddams, S. A.
Jenkins, Bethany D. *See* Armbrust, E. Virginia.
Jennerjahn, Tim C.; Ittekkot, Venugopalan; Arz, Helge W.; Behling, Hermann; Pätzold, Jürgen; Wefer, Gerold: Asynchronous terrestrial and marine signals of climate change during Heinrich events. p2236 24 Dec 2004.
Jentsch, Stefan. *See* Baumeister, Wolfgang.
Jerolmack, D. J. *See* Soderblom, L. A.
Jett, Bradley D. *See* Coburn, Phillip S.
Jetter, Reinhard. *See* Pighin, Jamie A.
Jetz, Walter; Carbone, Chris; Fulford, Jenny; Brown, James H.: The scaling of animal space use. p266 8 Oct 2004.
Ji, Hai. *See* Xia, Qingyou.
Jia, Jin-Feng. *See* Guo, Yang.
Jiang, D. *See* Novoselov, K. S.
Jianxiang, Yang (news): China takes bold steps into Antarctic's forbidding interior. p803 29 Oct 2004.
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Johnson, J. *See* Squyres, S. W.
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Kelsey, Harvey M. *See* Sawai, Yuki.
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Kerr, Richard A. *See* Pennisi, Elizabeth.
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Keyghobadi, Nusha. *See* Fonseca, Dina M.
Killingback, Timothy. *See* Doebeli, Michael.
Kim, Chanhong. *See* Wagner, Daniela.
Kim, D. S. *See* Bargheer, M.
Kim, Hackjin. *See* Whalen, Paul J.
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King, Randall W. *See* Verma, Rati.
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Kingston, Robert E. *See* Francis, Nicole J.
Kirby, Margaret L. *See* Wilbanks, Alyson M.
Kirk, Martin L. *See* Anderson, Travis M.
Kis-Varga, Miklós. *See* Erdélyi, Zoltán.
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Koenig, Jeremy E. *See* Papke, R. Thane.
Koetzle, Thomas F. *See* Anderson, Travis M.
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Kosintsev, Pavel. *See* Shapiro, Beth.
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Kovac, J. M. *See* Readhead, A. C. S.
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L and M

La Scola, Bernard. *See* Raoult, Didier.
Labarca, Cesar. *See* Tapper, Andrew R.
Labuda, Tord. *See* Gao, Min.
Lahankar, S. A. *See* Townsend, D.
Lahiri, Sukhamay. *See* Hoshi, Toshinori.
Lai, Barry. *See* Kemner, Kenneth M.
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Lawson, John A. *See* Egan, Karine M.
Lay, Thorne. *See* Garnero, Edward J.
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Leturcq, France. *See* Goyenvalle, Aurélie.
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Li, Hanchen. *See* Houghton, JeanMarie.
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Li, Jun. *See* Xia, Qingyou.
Li, R. *See* Arvidson, R. E. *See also* Bell, J. F., III. *See also* Soderblom, L. A. *See also* Squyres, S. W.
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Li, Xiao-Hong. *See* Leinders-Zufall, Trese.
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Lifton, Richard P. *See* Wilson, Frederick H.
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Lin, Ying. *See* Xia, Qingyou.
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Linet, Martha. *See* Lan, Qing.
Liolios, Konstantinos. *See* Hiller, N. Luisa.
Lippmeier, J. Casey. *See* Armbrust, E. Virginia.
Lira, Alena. *See* Ansorge, Mark S.
Litwin, Daryl E. *See* Knight, Alec.
Liu, Bin. *See* Xia, Qingyou.
Liu, Chun. *See* Xia, Qingyou.
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Liu, Y. *See* Nelson, K. D.
Liu, Yang. *See* Gray, Paul A.
Liu, Yun-Cai. *See* Gao, Min.
Livingston, Pat. *See* Pikitch, Ellen K.
Ljung, Karin. *See* Friml, Jiří.
Lloyd, Sarah E. *See* Wadsworth, Jonathan D. F.
Lloyd, Seth. *See* Giovannetti, Vittorio.
Lodge, Timothy P. *See* Li, Zhibo.
Loewenstein, George. *See* McClure, Samuel M.
Look, A. Thomas. *See* Weng, Andrew P.
Lopez-Estraño, Carlos. *See* Hiller, N. Luisa.
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Lu, Cheng. *See* Xia, Qingyou.
Lucas, Susan. *See* Armbrust, E. Virginia.
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Malakoff, David. *See* Kaiser, Jocelyn. *See also* Vogel, Gretchen.
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Malin, M. *See* Bell, J. F., III.
Malin, M. C. *See* Soderblom, L. A. *See also* Squyres, S. W.
Maller, James L. *See* Matsumoto, Yutaka.
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Manizade, S. *See* Thomas, R.
Manji, H. K. *See* Birnbaum, S. G.
Manley, Kate. *See* Elphick, Gwendolyn F.
Mannaert, An. *See* Bossuyt, Franky.
Manova, Katia. *See* Fraidenraich, Diego.
Manthe, Uwe. *See* Wu, Tao.
Mao, Bingyu. *See* Xia, Qingyou.
Mao, Z. Q. *See* Nelson, K. D.
Maor, Eli: review of π, by Alfred S. Posamentier and Ingmar Lehmann. p1894 10 Dec 2004.
Marcotte, Edward M. *See* Lee, Insuk.
Marcus-Samuels, Bernice. *See* Gershengorn, Marvin C.
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Marino, Ignazio R. and Cirillo, Claudia: Clinical trials or exploitation? (letter). p54 1 Oct 2004.
Marks, Michael J. *See* Tapper, Andrew R.
Marlovits, Thomas C.; Kubori, Tomoko; Sukhan, Anand; Thomas, Dennis R.; Galán, Jorge E.; Unger, Vinzenz M.: Structural insights into the assembly of the type III secretion needle complex. p1040 5 Nov 2004.
Marmer, Barry L. *See* Saffarian, Saveez.
Marra, Peter P. *See* Norris, D. Ryan.
Marsh, Kenneth N. *See* Florusse, Louw J.
Marshall, Eliot (news): Experts warn against censoring basic science. p2022 17 Dec 2004. Getting the noise out of gene arrays. p630 22 Oct 2004.
Marti, Matthias; Good, Robert T.; Rug, Melanie; Knuepfer, Ellen; Cowman, Alan F.: Targeting malaria virulence and remodeling proteins to the host erythrocyte. p1930 10 Dec 2004.
Martin, D. Christopher. *See* Friedman, Peter G.
Martin, Larry D. *See* Shapiro, Beth.
Martinek, Jan. *See* Pasupathy, Abhay N.
Martinez, Diego. *See* Armbrust, E. Virginia.
Martinez, Natalia. *See* Tomari, Yukihide.
Martins, Nathalie. *See* Espagne, Eric.
Martrat, Belen; Grimalt, Joan O.; Lopez-Martinez,

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Marty, Bernard. *See* Dauphas, Nicolas.
Marx, Jean (news): Bone marrow cells: the source of gastric cancer? p1455 26 Nov 2004. Inflammation and cancer: the link grows stronger. p966 5 Nov 2004. Metabolic defects tied to mitochondrial gene. p592 22 Oct 2004.
Maser, Joerg. *See* Kemner, Kenneth M.
Maskin, Eric: review of *Microeconomics*, by Samuel Bowles. p1293 19 Nov 2004.
Maslin, Mark: Ecological versus climatic thresholds (perspective). p2197 24 Dec 2004.
Mason, B. S. *See* Readhead, A. C. S.
Mason, Christopher. *See* Stolc, Viktor.
Mason, Helen S. *See* Williams, Sandile E. J.
Massire, Christian. *See* Westhof, Eric.
Matheus, Paul; Burns, James; Weinstock, Jaco; Hofreiter, Michael: Pleistocene brown bears in the mid-continent of North America. p1150 12 Nov 2004.
Matheus, Paul E. *See* Shapiro, Beth.
Matranga, Christian. *See* Tomari, Yukihide.
Matrosova, V. Y. *See* Daly, M. J.
Matsukevich, D. N. and Kuzmich, A.: Quantum state transfer between matter and light. p663 22 Oct 2004.
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Matter, Christian M. *See* Ricci, Romeo.
Mattner, Jochen. *See* Zhou, Dapeng.
Matuschka, F.-R. *See* Spielman, A.
Maul-Pavicic, Andrea. *See* Leinders-Zufall, Trese.
Maupin, Valérie. *See* Garnero, Edward J.
Maxwell, Jeffrey S. *See* Whalen, Paul J.
May, J. *See* Readhead, A. C. S.
Mays, Charlotte A. *See* Shimizu, Kentaro K.
McAllister, Murdoch. *See* Pikitch, Ellen K.
McCarthy, Thomas J. *See* Shin, Kyusoon.
McCartney, E. *See* Bell, J. F., III.
McClure, Samuel M.; Laibson, David I.; Loewenstein, George; Cohen, Jonathan D.: Separate neural systems value immediate and delayed monetary rewards. p503 15 Oct 2004.
McDonald, Charles H. *See* Wang, Yanming.
McDowell, H. P. *See* Nelson, D. E.
McEuen, Paul L. *See* Pasupathy, Abhay N.
McFall-Ngai, Margaret J. *See* Koropatnick, Tanya A.
McGrew, William C. *See* Brüne, Martin.
McKay, Christopher P. *See* Navarro-Gonzalez, Rafael.
McKinney, Sheri L. *See* Tapper, Andrew R.
McLaren, Donald G. *See* Whalen, Paul J.
McLean, Ashly A. *See* Whalen, Paul J.
McLennan, S. *See* Bell, J. F., III.
McLennan, S. M. *See* Herkenhoff, K. E. *See also* Soderblom, L. A. *See also* Squyres, S. W.
McMahon, Andrew P. *See* Gray, Paul A.
McMahon, Michelle M. *See* Driskell, Amy C.
McManamon, Francis P.: The next step for Kennewick Man (letter). p612 22 Oct 2004.
McPherson, John D. *See* Liao, Guochun.
McSween, H. Y. *See* Herkenhoff, K. E. *See also* Soderblom, L. A. *See also* Squyres, S. W.
McSween, H. Y., Jr. *See* Bell, J. F., III. *See also* Christensen, P. R. *See also* Squyres, S. W.
McVey Ward, Diane. *See* Nemeth, Elizabeta.
Medalia, Ohad. *See* Beck, Martin.
Medina, Mónica. *See* Armbrust, E. Virginia.
Meegaskumbura, Madhava. *See* Bossuyt, Franky.
Mees, Alexandra; Klar, Tobias; Gnau, Petra; Hennecke, Ulrich; Eker, Andre P. M.; Carell, Thomas; Essen, Lars-Oliver: Crystal structure of a photolyase bound to a CPD-like DNA lesion after in situ repair. p1789 3 Dec 2004.
Mefford, Megan. *See* Lederman, Michael M.
Mehall, G. L. *See* Christensen, P. R.
Mehall, L. K. *See* Christensen, P. R.
Mehlmann, Lisa M.; Saeki, Yoshinaga; Tanaka, Shigeru; Brennan, Thomas J.; Evsikov, Alexei V.; Pendola, Frank L.; Knowles, Barbara B.; Eppig, John J.; Jaffe, Laurinda A.: The G_s-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. p1947 10 Dec 2004.
Meister, Patrick. *See* D'Hondt, Steven.
Meitz, Julia C. *See* Allen, Rebecca L.
Meko, David M. *See* Cook, Edward R.
Melchior, Frauke. *See* Beck, Martin.
Melosh, H. Jay. *See* Renne, Paul R. *See also* Richardson, James E.
Meng, Anming. *See* Zhang, Lixia.
Meng, Qingshun. *See* Xia, Qingyou.
Mervis, Jeffrey (news): Advice on science advising leaves plenty of questions. p1450 26 Nov 2004. Breakdown of the year: the unwritten contract. p2015 17 Dec 2004. Bush victory leaves scars—and concerns about funding. p1110 12 Nov 2004. End of cost sharing could boost competition. p595 22 Oct 2004. Male sweep of new award raises questions of bias. p595 22 Oct 2004. Nominee scores Cabinet hat trick. p2018 17 Dec 2004. NSF blocked from funding Smithsonian scientists. p1873 10 Dec 2004. Risky business. p220 8 Oct 2004. Santa Fe Institute seeks president. p33 1 Oct 2004. What can NIH do for physicists? p1463 26 Nov 2004.
Mervis, Jeffrey. *See* Lawler, Andrew.
Messer, Benjamin M. *See* Smith, Jared D.
Metzger, A. E. *See* Sprague, A. L.
Meyer-Berthaud, B. *See* Gerrienne, P.
Meyers, Lauren Ancel: review of *Modularity in Development and Evolution*, by Gerhard Schlosser and Günter P. Wagner, Eds. p814 29 Oct 2004.
Meyron-Holtz, Esther G.; Ghosh, Manik C.; Rouault, Tracey A.: Mammalian tissue oxygen levels modulate iron-regulatory protein activities in vivo. p2087 17 Dec 2004.
Michalakis, Yannis and Roze, Denis: Epistasis in RNA viruses (perspective). p1492 26 Nov 2004.
Michniewicz, Marta. *See* Friml, Jiří.
Milhollin, Gary. *See* Palmer, Liz.
Milinkovitch, Michel C. *See* Bossuyt, Franky.
Miller, D. Jay. *See* D'Hondt, Steven.
Miller, Greg (news): Axel, Buck share award for deciphering how the nose knows. p207 8 Oct 2004. Behavioral neuroscience uncaged. p432 15 Oct 2004. Brain cells may pay the price for a bad night's sleep. p1126 12 Nov 2004. Listen, baby. p1127 12 Nov 2004.
Milne, Jennifer. *See* Somerville, Chris.
Minelli, Alessandro: Bits and pieces (perspective). p1693 3 Dec 2004.
Ming, D. W. *See* Arvidson, R. E. *See also* Bell, J. F., III. *See also* Herkenhoff, K. E. *See also* Klingelhöfer, G. *See also* Rieder, R. *See also* Soderblom, L. A. *See also* Squyres, S. W.
Minton, Nigel. *See* Nioche, Pierre.
Mitchison, Timothy J. *See* Perlman, Zachary E.
Mitchnik, Mark. *See* Coplan, Paul M.
Mitterer, Richard. *See* D'Hondt, Steven.
Miyashita, Yasushi: Cognitive memory: cellular and network machineries and their top-down control. p435 15 Oct 2004.
Miyawaki, Atsushi. *See* Ando, Ryoko.
Mizuno, Hideaki. *See* Ando, Ryoko.
Mizuno, Kohei. *See* Hata, Kenji.
Mizushima, Noboru. *See* Nakagawa, Ichiro.
Mizutani, Takeshi. *See* Katayama, Yoshinori.
Moersch, J. *See* Squyres, S. W.
Moersch, J. E. *See* Christensen, P. R.
Mogi, Motoyoshi. *See* Fonseca, Dina M.
Mohd-Sarip, Adone and Verrijzer, C. Peter: A higher order of silence (perspective). p1484 26 Nov 2004.
Mojzsis, Stephen J. *See* Turner, Grenville.
Møller, Jesper Vuust. *See* Olesen, Claus.
Møller-Jensen, Jakob and Gerdes, Kenn: Dynamic instability of a bacterial engine (perspective). p987 5 Nov 2004.
Mondragón, Alfonso. *See* Krasilnikov, Andrey S.
Moniz, Ernest. *See* Deutch, John.
Monterosso, John. *See* Ainslie, George.
Montgomerie, Robert. *See* Norris, D. Ryan.
Montsant, Anton. *See* Armbrust, E. Virginia.
Moon, Sung In. *See* Shin, Kyusoon.
Moore, Kathryn J. *See* Ricci, Romeo.
Moore, LeRoy: Remediating Rocky Flats (letter). p975 5 Nov 2004.
Moore, Michael J. and Early, Greg A.: Cumulative sperm whale bone damage and the bends. p2215 24 Dec 2004.
Moore, Richard C. *See* Shimizu, Kentaro K.
Moran, Nancy A. *See* Daubin, Vincent.
Morbidelli, Alessandro: How Neptune pushed the boundaries of our Solar System (perspective). p1302 19 Nov 2004.
Moretti, Alessandra. *See* Chien, Kenneth R.
Moritz, Max A. and Odion, Dennis C.: Prescribed fire and natural disturbance (letter). p1680 3 Dec 2004.
Morokuma, Keiji. *See* Anderson, Travis M.
Morozov, S. V. *See* Novoselov, K. S.
Morris, John P., IV. *See* Weng, Andrew P.
Morris, R. V. *See* Bell, J. F., III. *See also* Christensen, P. R. *See also* Klingelhöfer, G. *See also* Soderblom, L. A. *See also* Squyres, S. W.
Morrison, Michael. *See* Deardorff, Don.
Morus, Iwan Rhys: review of *Promethean Ambitions*, by William R. Newman. p59 1 Oct 2004.
Mosier, Donald E. *See* Lederman, Michael M.
Moyà-Solà, Salvador; Köhler, Meike; Alba, David M.; Casanovas-Vilar, Isaac; Galindo, Jordi: *Pierolapithecus catalaunicus*, a new Middle Miocene great ape from Spain. p1339 19 Nov 2004.
Mu, James. *See* Kong, Mei.
Müller, Ulrike K. and Lentink, David: Turning on a dime (perspective). p1899 10 Dec 2004.
Mullins, R. Dyche. *See* Garner, Ethan C.
Munstermann, L. E. *See* Spielman, A.
Muoio, Deborah M. and Newgard, Christopher B.: Insulin resistance takes a trip through the ER (perspective). p425 15 Oct 2004.
Murray, Richard W.: A bit of history about Edwin A. Link (letter). p57 1 Oct 2004.
Musaev, Djamaladdin G. *See* Anderson, Travis M.
Myers, R. C. *See* Kato, Y. K.
Myers, S. T. *See* Readhead, A. C. S.
Myneni, Satish C. B. *See* Maria, Steven F.
Myrick, T. *See* Arvidson, R. E.

N, O, and P

Nabi, Ivan R. *See* Partridge, Emily A.
Nadkarni, Nalini M. *See* Bawa, Kamaljit S.
Naehr, Thomas. *See* D'Hondt, Steven.
Nagumo, Tamotsu. *See* Sawai, Yuki.
Nair, Shalini. *See* Roper, Cally.
Nairn, A. C. *See* Rakhilin, S. V.
Nakagawa, Ichiro; Amano, Atsuo; Mizushima, Noboru; Yamamoto, Akitsugu; Yamaguchi, Hitomi; Kamimoto, Takahiro; Nara, Atsuki; Funao, Junko; Nakata, Masanobu; Tsuda, Kayoko; Hamada, Shigeyuki; Yoshimori, Tamotsu: Autophagy defends cells against invading group A *Streptococcus*. p1037 5 Nov 2004.
Nakata, Masanobu. *See* Nakagawa, Ichiro.
Namai, Tatsunori. *See* Hata, Kenji.
Nara, Atsuki. *See* Nakagawa, Ichiro.
Nasci, R. S. *See* Spielman, A.
Nashmi, Raad. *See* Tapper, Andrew R.
Nasu, Hiroo. *See* Sawai, Yuki.
Nater, Mena. *See* Wagner, Daniela.
Naumova, N. B. *See* Dolfing, J.

Navarro-Gonzalez, Rafael; Rainey, Fred A.; McKay, Christopher P.: Microbial life in the Atacama Desert (letter). p1289 19 Nov 2004.
Nealson, Kenneth H. *See* Kemner, Kenneth M.
Neilson, J. W. *See* Maier, R. M.
Neiwert, Wade A. *See* Anderson, Travis M.
Nelson, Christopher D. *See* Chen, Wei.
Nelson, D. E.; Ihekwaba, A. E. C.; Elliott, M.; Johnson, J. R.; Gibney, C. A.; Foreman, B. E.; Nelson, G.; See, V.; Horton, C. A.; Spiller, D. G.; Edwards, S. W.; McDowell, H. P.; Unitt, J. F.; Sullivan, E.; Grimley, R.; Benson, N.; Broomhead, D.; Kell, D. B.; White, M. R. H.: Oscillations in NF-κB signaling control the dynamics of gene expression. p704 22 Oct 2004.
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Nelson, G. *See* Nelson, D. E.
Nelson, K. D.; Mao, Z. Q.; Maeno, Y.; Liu, Y.: Odd-parity superconductivity in Sr_2RuO_4. p1151 12 Nov 2004.
Nelson-Williams, Carol. *See* Wilson, Frederick H.
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Neuberger, Georg. *See* Zimmermann, Robert.
Neumark, D. M. *See* Bragg, A. E.
Nevitt, Gabrielle A. *See* Bonadonna, Francesco.
Newgard, Christopher B. *See* Muoio, Deborah M.
Newman, D. K. *See* Malasarn, D.
Newman, Paul. *See* Ehret, Christopher.
News Staff, the (news): Areas to watch in 2005. p2014 17 Dec 2004. The runners-up. p2013 17 Dec 2004. Scorecard 2003. p2012 17 Dec 2004.
Newsom, Gerald H.: Tuition costs and funding needy students (letter). p811 29 Oct 2004.
Newsome, Timothy P.; Scaplehorn, Niki; Way, Michael: Src mediates a switch from microtubule- to actin-based motility of vaccinia virus. p124 1 Oct 2004.
Ng, Anh. *See* Liao, Guochun.
Ng, Peter K. L. *See* Bossuyt, Franky.
Ni, Peixiang. *See* Xia, Qingyou.
Nicholson, C. *See* Becker, L.
Nielsen, Rikke Christina. *See* Olesen, Claus.
Nieuwenhuis, Sander. *See* Ridderinkhof, K. Richard.
Niitsuma, Sachiko. *See* D'Hondt, Steven.
Nilsson, Göran E. *See* Stecyk, Jonathan A. W.
Ning, Yuanheng. *See* Zhang, Lixia.
Nioche, Pierre; Berka, Vladimir; Vipond, Julia; Minton, Nigel; Tsai, Ah-Lim; Raman, C. S.: Femtomolar sensitivity of a NO sensor from *Clostridium botulinum*. p1550 26 Nov 2004.
Nishi, A. *See* Rakhilin, S. V.
Nissen, Poul. *See* Olesen, Claus.
Niu, Qian. *See* Guo, Yang.
Nix, William D. *See* Uchic, Michael D.
Noe Dobrea, E. Z.. *See* Bell, J. F., III.
Nomura, Sachiyo. *See* Houghton, JeanMarie.
Norman, Marc D. *See* Humayun, Munir.
Normile, Dennis (news): Asia struggles to keep humans and chickens apart. p399 15 Oct 2004. Consortium hopes to map human history in Asia. p1667 3 Dec 2004. Ducks may magnify threat of avian flu virus. p953 5 Nov 2004. Expanding trade with China creates ecological backlash. p968 5 Nov 2004. Japan to host global forum on societal impacts. p959 5 Nov 2004. Some countries are betting that a few seconds can save lives. p2178 24 Dec 2004. Vaccinating birds may help to curtail virus's spread. p398 15 Oct 2004.
Norris, D. E. *See* Spielman, A.
Norris, D. Ryan; Marra, Peter P.; Montgomerie, Robert; Kyser, T. Kurt; Ratcliffe, Laurene M.: Reproductive effort, molting latitude, and feather color in a migratory songbird. p2249 24 Dec 2004.
North, Gerald. *See* Foukal, Peter.
Nosten, François. *See* Roper, Cally.
Nottenburg, Carol. *See* Koo, Bonwoo.
Notterman, Joseph M.: The brain, neurons, and behavior (letter). p1683 3 Dec 2004.
Novak, R. J. *See* Spielman, A.
Novoselov, K. S.; Geim, A. K.; Morozov, S. V.; Jiang, D.; Zhang, Y.; Dubonos, S. V.; Grigorieva, I. V.; Firsov, A. A.: Electric field effect in atomically thin carbon films. p666 22 Oct 2004.
Nowotny, Helga. *See* Krull, Wilhelm.
Nunès, Jacques A. *See* Vivier, Eric.
Oates, C. W. *See* Diddams, S. A.
Obornik, Miroslav. *See* Armbrust, E. Virginia.
O'Brien, Stephen P. *See* Sfeir, Matthew Y.
Odion, Dennis C. *See* Moritz, Max A.
Offord, Robin. *See* Lederman, Michael M.
Offringa, Remko. *See* Friml, Jiří.
Ogata, Hiroyuki. *See* Raoult, Didier.
Okeke, Iruka N.: Stopping the spread of drug-resistant malaria (letter). p2039 17 Dec 2004.
Okubo, Shuhei. *See* Imanishi, Yuichi.
Olesen, Claus; Sørensen, Thomas Lykke-Møller; Nielsen, Rikke Christina; Møller, Jesper Vuust; Nissen, Poul: Dephosphorylation of the calcium pump coupled to counterion occlusion. p2251 24 Dec 2004.
Oliveri, Paola. *See* Chen, Jun-Yuan.
O'Loughlin, Edward J. *See* Kemner, Kenneth M.
Olsen, Kenneth M. *See* Shimizu, Kentaro K.
Olson, L. E.; Richtsmeier, J. T.; Leszl, J.; Reeves, R. H.: A chromosome 21 critical region does not cause specific Down syndrome phenotypes. p687 22 Oct 2004.
Olson, P. A. *See* Rakhilin, S. V.
O'Meara, Brian C. *See* Driskell, Amy C.
Omelchenko, M. V. *See* Daly, M. J.
Omura, Kentaro. *See* Yamashita, Futoshi.
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Oommen, Oommen V. *See* Bossuyt, Franky.
op den Camp, Roel. *See* Wagner, Daniela.
O'Rand, M. G.; Widgren, E. E.; Sivashanmugam, P.; Richardson, R. T.; Hall, S. H.; French, F. S.; VandeVoort, C. A.; Ramachandra, S. G.; Ramesh, V.; Jagannadha Rao, A.: Reversible immunocontraception in male monkeys immunized with Eppin. p1189 12 Nov 2004.
O'Reilly, Randall C. *See* Frank, Michael J.
Oreskes, Naomi: The scientific consensus on climate change (essays on science and society). p1686 3 Dec 2004.
Orgel, Leslie. *See* Leman, Luke.
Oroudjev, Emin. *See* Chworos, Arkadiusz.
Osborn, Timothy J. and Briffa, Keith R.: The real color of climate change? (perspective). p621 22 Oct 2004.
Osborne, Erin. *See* Somerville, Chris.
Osborne, Ian and Clery, Daniel (news): Higher standards. p1307 19 Nov 2004.
Osterhaus, Albert. *See* Kuiken, Thijs.
Osterwalder, J. *See* Baumberger, F.
Ostfeld, Richard S. and Keesing, Felicia: Oh the locusts sang, then they dropped dead (perspective). p1488 26 Nov 2004.
Ouwerkerk, Pieter B. F. *See* Friml, Jiří.
Özcan, Umut; Cao, Qiong; Yilmaz, Erkan; Lee, Ann-Hwee; Iwakoshi, Neal N.; Özdelen, Esra; Tuncman, Gürol; Görgün, Cem; Glimcher, Laurie H.; Hotamisligil, Gökhan S.: Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. p457 15 Oct 2004.
Özdelen, Esra. *See* Özcan, Umut.
Ozin, Geoffrey A. *See* Landskron, Kai.
Padilla, Christian Naranjo. *See* D'Hondt, Steven.
Padin, S. *See* Readhead, A. C. S.
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Palumbo, Giorgio G. C. *See* Grandi, Paola.
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Parker, T. J. *See* Bell, J. F., III.
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Pendola, Frank L. *See* Mehlmann, Lisa M.
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Penner, Reinhold. *See* Launay, Pierre.
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Perham, Richard N. *See* Frank, René A. W.
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Perlin, Julie R. *See* Wang, Yanming.
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Persson, Staffan. *See* Somerville, Chris.
Peters, Cor J. *See* Florusse, Louw J.
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Pillar, Christopher M. *See* Coburn, Phillip S.
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Plitzko, Jürgen M. *See* Beck, Martin.
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Pogosyan, D. *See* Readhead, A. C. S.
Poirié, Marylène. *See* Espagne, Eric.
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Pope, K. O. *See* Becker, L.
Poreda, R. J. *See* Becker, L.
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Pospieszalski, M. *See* Readhead, A. C. S.
Poulin, P. *See* Loudet, J. C.
Povel, G. D. E. *See* Videler, J. J.
Powelson, Julie. *See* Nemeth, Elizabeta.
Pratap, J. Venkatesh. *See* Frank, René A. W.
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Proia, Richard L. *See* Zhou, Dapeng.
Proton, J. *See* Bell, J. F., III.
Provost, Bertille. *See* Espagne, Eric.
Pryke, C. *See* Readhead, A. C. S.
Przyborski, Jude and Lanzer, Michael: The malarial secretome (perspective). p1897 10 Dec 2004.
Przybyla, Dominika. *See* Wagner, Daniela.
Puech, Anne. *See* Liao, Guochun.
Purugganan, Michael D. *See* Shimizu, Kentaro K.
Putnam, Nicholas H. *See* Armbrust, E. Virginia.
Pybus, Oliver G. *See* Shapiro, Beth.
Pyo, Tae-Soo. *See* Takato, Naruhisa.

Q and R

Qi, Kai. *See* Pochan, Darrin J.
Qi, Qiuhui. *See* Xia, Qingyou.
Qian, Jifeng. *See* Xia, Qingyou.
Qin, Liping. *See* Humayun, Munir.
Qiu, Z. Q. *See* Guo, Yang.
Quade, J. *See* Maier, R. M.
Querbes, William. *See* Elphick, Gwendolyn F.
Quigg, Antonietta. *See* Grzebyk, Daniel.
Quint, Ab. *See* Friml, Jiří.
Raab, Ted. *See* Somerville, Chris.
Raaka, Bruce M. *See* Gershengorn, Marvin C.
Rabkin, Charles. *See* Lan, Qing.
Rader, Daniel J. *See* Egan, Karine M.
Radloff, Wolfgang. *See* Schultz, Thomas.
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Raine, Cedric S. *See* Arnett, Heather A.
Rainey, Fred A. *See* Navarro-Gonzalez, Rafael.
Raja, Khalid M. *See* Wilson, Frederick H.
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Raman, C. S. *See* Nioche, Pierre.
Rambaut, Andrew. *See* Shapiro, Beth.
Ramesh, V. *See* O'Rand, M. G.
Rampino, Michael R. *See* Renne, Paul R.
Random Samples: Academia as "one-party" system. p1678 3 Dec 2004. AIDS art in Paris. p1129 12 Nov 2004. Apes for a cause. p225 8 Oct 2004. Bedside to bench. p1889 10 Dec 2004. Betting on Bush. p1129 12 Nov 2004. Bioinformatics award. p1471 26 Nov 2004. Birds attack cats. p808 29 Oct 2004. Britain over 3 billion years. p605 22 Oct 2004. Brits and bikes. p1888 10 Dec 2004. Catch them young. p1287 19 Nov 2004. Chimp the toolmaker. p403 15 Oct 2004. Cinema vérité. p607 22 Oct 2004. Civilian space race. p224 8 Oct 2004. Clam diet. p605 22 Oct 2004. Compassionate science. p45 1 Oct 2004. Conservation cliffhanger. p2036 17 Dec 2004. Cracking the code. p973 5 Nov 2004. Cruel irony. p809 29 Oct 2004. Crying a bad omen? p971 5 Nov 2004. Culture shift. p1471 26 Nov 2004. Data deluged. p1287 19 Nov 2004. Dead on. p1287 19 Nov 2004. The definitive Mozart. p1129 12 Nov 2004. Deliver us from evil. p2036 17 Dec 2004. Descartes winners. p1889 10 Dec 2004. Developmental engineering. p225 8 Oct 2004. Eclipse close-up. p1888 10 Dec 2004. Eliminating hoops. p809 29 Oct 2004. EMBO prize. p225 8 Oct 2004. Engineering prizes. p607 22 Oct 2004. Expectant fish. p403 15 Oct 2004. Explorer of the unknown. p405 15 Oct 2004. Failed initiative. p45

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Reeves, R. *See* Readhead, A. C. S.

Reeves, R. H. *See* Olson, L. E.

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Reiche, P. *See* Choi, K. J.

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Reisen, W. K. *See* Spielman, A.

Reiter, P. *See* Spielman, A.

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Renz, F. *See* Klingelhöfer, G.

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Resovsky, Joseph. *See* Trampert, Jeannot.

Rheinecker, J. *See* Townsend, D.

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Riccardi, Daniela. *See* Williams, Sandile E. J.

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Richmond, Timothy J. *See* Dorigo, Benedetta.

Richter, L. *See* Arvidson, R. E. *See also* Herkenhoff, K. E. *See also* Soderblom, L. A. *See also* Squyres, S. W.

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Riederer, Monika. *See* Zimmermann, Robert.

Rifkin, Scott A. *See* Stolc, Viktor.

Rignot, E. *See* Thomas, R.

Rim, Ken. *See* Ieong, Meikei.

Rimmelzwaan, Guus. *See* Kuiken, Thijs.

Rinn, John L. *See* Bertone, Paul.

Ritchie, R. O. *See* Ziegler, A.

Rivera, A. *See* Thomas, R.

Robert, Catherine. *See* Raoult, Didier.

Roberts, Leslie. *See* Jasny, Barbara R.

Rodionov, D. *See* Soderblom, L. A.

Rodionov, D. S. *See* Klingelhöfer, G.

Rodrigues, Ana S. L. *See* Stuart, Simon N.

Rodriguez, Benigno. *See* Lederman, Michael M.

Rodríguez-Valera, Francísco. *See* Papke, R. Thane.

Roe, Dilys. *See* Adams, William M.

Roeder, Robert G. *See* Wang, Yanming.

Roelants, Kim. *See* Bossuyt, Franky.

Rogers, A. D. *See* Christensen, P. R.

Rogers, Arlin B. *See* Houghton, JeanMarie.

Rokhsar, Daniel S. *See* Armbrust, E. Virginia.

Romero, Elizabeth. *See* Fraidenraich, Diego.

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Roscioli, Joseph R. *See* Hammer, Nathan I.

Rose, Laura E. *See* Allen, Rebecca L.

Rosenberg, Zeda F. *See* Coplan, Paul M.

Rosenzweig, Robert M.: Controversies over tuition increases (letter). p811 29 Oct 2004.

Rosseinsky, Matthew J. *See* Zhao, Xuebo.

Roth, Bryan L. *See* Elphick, Gwendolyn F.

Rothman, Nathaniel. *See* Lan, Qing.

Rouault, Tracey A. *See* Meyron-Holtz, Esther G.

Roucoux, K. H. *See* Tzedakis, P. C.

Rowan, Linda (news): Opportunity runneth over. p1697 3 Dec 2004.

Rowitch, David H. *See* Arnett, Heather A. *See also* Gray, Paul A.

Royce, Thomas *See* Bertone, Paul. *See also* Hall, David A.

Roze, Denis. *See* Michalakis, Yannis.

Rozenberg, Izabela. *See* Ricci, Romeo.

Rozowsky, Joel S. *See* Bertone, Paul.

Rubin, Vera: Neglect of women in science (letter). p1891 10 Dec 2004.

Ruesink, Jennifer L. *See* Solan, Martin.

Ruff, S. W. *See* Christensen, P. R.

Rug, Melanie. *See* Marti, Matthias.

Rushworth, Linda. *See* O'Neill, Eric.

Russell, Lynn M. *See* Maria, Steven F.

Russell, R. *See* Thomas, R.

Russell, Thomas P. *See* Shin, Kyusoon.

Rustichini, Aldo. *See* Glimcher, Paul W.

Rutherford, Scott D. *See* D'Hondt, Steven.

Ruzsa, Stephanie. *See* Shimizu, Kentaro K.

Ruzzo, Walter L. *See* Mandal, Maumita.

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Rynearson, Tatiana A. *See* Armbrust, E. Virginia.

S

S., Prashanth Chandramani. *See* Leinders-Zufall, Trese.

Sablowski, R. *See* Gazzani, S.

Saeki, Yoshinaga. *See* Mehlmann, Lisa M.

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Sainsbury, Keith. *See* Pikitch, Ellen K.
Saito, Mak A. *See* Armbrust, E. Virginia.
Sakakibara, T. *See* Grigera, S. A.
Sakamoto, Kathleen M. *See* Verma, Rati.
Sakata, Kazuko. *See* Pang, Petti T.
Sakmann, Bert. *See* Petersen, Carl C. H.
Salkowitz, Janelle R. *See* Lederman, Michael M.
Saltikov, C. W. *See* Malasarn, D.
Sam, Moses K. *See* Brashares, Justin S.
Samanta, Manoj. *See* Bertone, Paul.
Samoylova, Elena. *See* Schultz, Thomas.
Samuels, A. Lacey. *See* Pighin, Jamie A.
Sanchez-Irizarry, Cheryll. *See* Weng, Andrew P.
Sandberg, Göran. *See* Friml, Jiří.
Sanderson, Michael J. *See* Driskell, Amy C.
Santini, J. M. *See* Malasarn, D.
Santora, Christine. *See* Pikitch, Ellen K.
Sasaki, Jun. *See* Vogeley, Lutz.
Sass, Henrik. *See* D'Hondt, Steven.
Satake, Kenji. *See* Sawai, Yuki.
Sato, Tadahiro. *See* Imanishi, Yuichi.
Savage, H. M. *See* Spielman, A.
Savage, Martha K. *See* Gerst, Alexander.
Savage, Paul B. *See* Zhou, Dapeng.
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Ullsperger, Markus. *See* Ridderinkhof, K. Richard.
Unger, Vinzenz M. *See* Marlovits, Thomas C.
Unitt, J. F. *See* Nelson, D. E.
Urban, Alexander E. *See* Bertone, Paul.
Usuka, Jonathan. *See* Liao, Guochun.
Utsumi, Wataru. *See* Katayama, Yoshinori.
Valentin, Klaus. *See* Armbrust, E. Virginia.
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van Amerongen, Geert. *See* Kuiken, Thijs.
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van Riel, Debby. *See* Kuiken, Thijs.
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Varadan, Ranjani. *See* Verma, Rati.
Vardi, Assaf. *See* Armbrust, E. Virginia.
Vasilenko, A. *See* Daly, M. J.
Vaughn, Michael B. *See* Nemeth, Elizabeta.
Veazey, Ronald S. *See* Lederman, Michael M.
Vély, Frédéric. *See* Vivier, Eric.
Venkateswaran, A. *See* Daly, M. J.
Venkitaraman, Ashok R. *See* Daniels, Matthew J.
Verdun, Ramiro E. *See* Crabbe, Laure.
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Verrijzer, C. Peter. *See* Mohd-Sarip, Adone.
Viciani, Silvia. *See* Zavatta, Alessandro.
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Vos, A. *See* Dolfing, J.
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Waller, Robert W. *See* Stuart, Simon N.
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Wan, Yongji. *See* Xia, Qingyou.
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Wang, E. G. *See* Guo, Yang.
Wang, Feng. *See* Sfeir, Matthew Y.
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Wang, Timothy C. *See* Houghton, JeanMarie.
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Wänke, H. *See* Rieder, R. *See also* Squyres, S. W.
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Washburn, Michael P. *See* Kusch, Thomas.
Watling, John. *See* Wignall, Paul.
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Watters, W. A. *See* Soderblom, L. A.
Way, Michael. *See* Newsome, Timothy P.
Wdowiak, T. *See* Herkenhoff, K. E. *See also* Klingelhöfer, G. *See also* Squyres, S. W.
Webb, Watt W. *See* Kasischke, Karl A.
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Wefer, Gerold. *See* Jennerjahn, Tim C.
Wegener, Martin. *See* Linden, Stefan.
Wei, Chiju. *See* Gershengorn, Marvin C.
Wei, Ning. *See* Xia, Qingyou.
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Weijers, Dolf. *See* Friml, Jiří.
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Weinberg, Zasha. *See* Mandal, Maumita.
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Weinkam, Patrick. *See* Chworos, Arkadiusz.
Weinstock, Jaco. *See* Matheus, Paul.
Weissman, Allan. *See* Baumeister, Wolfgang.
Weissman, Sherman. *See* Bertone, Paul.
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Weitz, C. M. *See* Bell, J. F., III. *See also* Soderblom, L. A.
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Whitcomb, Jeanette M. *See* Bonhoeffer, Sebastian.
White, Kevin P. *See* Stolc, Viktor.
White, M. R. H. *See* Nelson, D. E.
White, P. Scott. *See* Knight, Alec.
Whiteaker, Paul. *See* Tapper, Andrew R.
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Whittemore, Alice S. *See* Easton, Douglas F.
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Widmayer, Patricia. *See* Leinders-Zufall, Trese.
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Wittbrodt, Joachim. *See* Arendt, Detlev.
Witvrouw, Ann. *See* Van Hoof, Chris.
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Wolf, Dieter. *See* Baumeister, Wolfgang.
Wolf, George H. *See* Yarger, Jeff L.
Wolff, M. *See* Squyres, S. W.
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Wolmer, William. *See* Adams, William M.
Wong, Gane Ka-Shu. *See* Xia, Qingyou.
Woo, J. C. *See* Bargheer, M.
Woo, Newton T. *See* Pang, Petti T.
Wood, William B. *See* Handelsman, Jo.
Woodcock, Christopher L. *See* Francis, Nicole J.
Woodhouse, Connie A. *See* Cook, Edward R.
Woodward, C. *See* Gazzani, S.
Woodward, James. *See* Adolphs, Ralph.
Wooley, Karen L. *See* Pochan, Darrin J.
Wootton, Phillippa. *See* Williams, Sandile E. J.
Workman, Jerry L. *See* Kusch, Thomas.
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Würsch, Marco. *See* Wagner, Daniela.

Wyatt, M. B. *See* Christensen, P. R. *See also* Soderblom, L. A.
Wysocka, Joanna. *See* Wang, Yanming.

X, Y, and Z

Xi, Yan. *See* Xia, Qingyou.
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Xia, Ying. *See* Gao, Min.
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Xiang, Zhonghuai. *See* Xia, Qingyou.
Xiao, Bo. *See* Zhao, Xuebo.
Xiao, Yinghua. *See* Krasilnikov, Andrey S.
Xu, Anne. *See* Kong, Mei.
Xu, Hanfu. *See* Xia, Qingyou.
Xu, Jianzhe. *See* Xia, Qingyou.
Xu, Jinshan. *See* Xia, Qingyou.
Xue, Qi-Kun. *See* Guo, Yang.
Yamaguchi, Hitomi. *See* Nakagawa, Ichiro.
Yamaguchi, Masaaki. *See* Sawai, Yuki.
Yamakata, Masaaki. *See* Katayama, Yoshinori.
Yamamoto, Akitsugu. *See* Nakagawa, Ichiro.
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Yamashita, Tadashi. *See* Zhou, Dapeng.
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Yang, Ding-Shyue. *See* Paik, D. Hern.
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Yang, Huanming. *See* Xia, Qingyou.
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Yang, Zheng. *See* Xia, Qingyou.
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Yates, John R., III. *See* Kusch, Thomas.
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Ye, Jia. *See* Xia, Qingyou.
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Ye, Jun. *See* Marian, Adela.
Yeates, Todd O. *See* Bowers, Peter M.
Yen, A. *See* Klingelhöfer, G. *See also* Rieder, R. *See also* Soderblom, L. A. *See also* Squyres, S. W.
Yilmaz, Erkan. *See* Özcan, Umut.
Yin, Ning. *See* Zhou, Dapeng.
Yin, Songnian. *See* Lan, Qing.
Yin, Yadong. *See* Son, Dong Hee.
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York, John D. and Hunter, Tony: Unexpected mediators of protein phosphorylation (perspective). p2053 17 Dec 2004.
Yoshimori, Tamotsu. *See* Nakagawa, Ichiro.
Young, Bruce E. *See* Stuart, Simon N.
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Young, S. Stanley and Bang, Heejun: The file-drawer problem, revisited (letter). p1133 12 Nov 2004.
Youngs, Heather. *See* Somerville, Chris.
Yu, Hong. *See* Xia, Qingyou.
Yu, Jing. *See* Gray, Paul A.
Yu, Jun. *See* Xia, Qingyou.
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Yuen, Dave. *See* Trampert, Jeannot.
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Yumura, Motoo. *See* Hata, Kenji.
Yung, Wing-Ho. *See* Pang, Petti T.
Yungel, J. *See* Thomas, R.
Zahn, Rainer. *See* Martrat, Belen.
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Zewail, Ahmed H. *See* Paik, D. Hern.
Zha, Xingfu. *See* Xia, Qingyou.
Zhai, M. *See* Daly, M. J.
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Zhang, Xiaowei. *See* Xia, Qingyou.
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Zhang, Yan-Feng. *See* Guo, Yang.
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Zhang, Yu. *See* Zhang, Lixia.
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Zhao, Chao. *See* Arnett, Heather A.
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